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(54) **PROCESS FOR PREPARING AMINO ACID.**

(57) A process for preparing amino acid, which comprises culturing transformed strain obtained by transforming host strain selected from microorganisms to Corynebacterium or Brevibacterium using recombinant DNA between a DNA fragment containing genes relating to an amino acid biosynthesis and vector DNA to accumulate the amino acid in the culture liquor, and recovering the amino acid.

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SPECIFICATION

PROCESS FOR PREPARING AMINO ACID

Technical Field

This invention relates to a process for producing amino acids by a novel expression method of a gene. More specifically, the present invention relates to a process for producing an amino acid by transforming a host microorganism belonging to the genus Corynebacterium or Brevibacterium with a recombinant DNA of a DNA fragment containing a gene involved in the biosynthesis of an amino acid and a vector DNA, culturing the transformant in a nutrient medium, accumulating the amino acid in the culture medium and recovering the amino acid therefrom.

Background Art

For the direct production of amino acids by fermentation, methods using wild-type strains, or mutant strains such as auxotrophic strains and amino acids analog-resistant strains, of the bacteria belonging to the genus Corynebacterium, Brevibacterium, Serratia, and the like are known.

For example, production of histidine using a strain resistant to histidine analog (Japanese Patent Publication No. 8596/81), production of tryptophan using a strain resistant to tryptophan analog (Japanese Patent Publication Nos. 4505/72 and 19037/76), production of isoleucine using a strain resistant to isoleucine analog (Japanese Patent Publication Nos. 38995/72, 6237/76 and 32070/79), production of phenylalanine using a strain resistant to phenylalanine analog (Japanese Patent Publication No. 10035/81), production of tyrosine using a strain requiring phenylalanine for its growth or being resistant to tyrosine [Agr. Chem. Soc., Japan 50 (1) R79-R87 (1976)], production of arginine using a strain resistant to L-arginine analog [Agr. Biol. Chem., 36, 1675-1684 (1972), Japanese Patent Publication Nos. 37235/79 and 150381/82] and the like are known.

Disclosure of the Invention

The present inventors have studied the production of an amino acid using a microorganism belonging to the genus Corynebacterium or Brevibacterium by recombinant DNA technology different from the conventional mutational breeding technology for the purpose of improving the amino acid productivity.

The present inventors have constructed plasmid vectors autonomously replicable in a microorganism belonging to the genus Corynebacterium or Brevibacterium and having selectable markers and adequate cloning sites and have developed a highly efficient transformation system (Japanese Published Unexamined Patent Application Nos. 183799/82, 186492/82 and 186489/82). Further, the present inventors have found that the plasmid vectors are useful for expressing a foreign gene in a host microorganism and increasing the productivity of amino acids by ligating a DNA fragment containing a foreign gene involved in the biosynthesis of amino acids such as glutamic acid and lysine to the plasmid vectors according to the procedures in recombinant DNA technology (Methods in Enzymology 68, Recombinant DNA, edited by Ray Wu, Academic Press 1979, U.S. Patent No. 4,237,224) and transforming Corynebacterium glutamicum L-22 or its derivatives using the transformation methods developed by the present inventors (Japanese Published Unexamined Patent Application No. 126789/83).

Furthermore, the present inventors have found that microorganisms prepared by the same method have acquired an increased productivity of histidine, tryptophan, phenylalanine, isoleucine, tyrosine and arginine and completed the present invention.

No example of expressing a desired gene and increasing productivity of an amino acid in a host microorganism belonging to the genus Corynebacterium or Brevibacterium by introducing a recombinant DNA containing such desired gene and vector, one of which is foreign to a host microorganism, into such host microorganism has been reported.

The present invention is explained in detail below.

The present invention provides a process for producing

an amino acid by cultivating in a medium a transformant which is obtained by transforming a microorganism belonging to the genus Corynebacterium or Brevibacterium with a recombinant DNA of a DNA fragment containing a gene involved in the biosynthesis of the amino acid and a vector DNA.

As the amino acid of the present invention, histidine, tryptophan, phenylalanine, isoleucine, tyrosine and arginine are mentioned.

As the DNA fragment containing the gene used in the present invention, the DNA fragment containing a gene involved in the biosynthesis of the amino acid of the present invention derived from eukaryotes, prokaryotes, viruses, bacteriophages or plasmids is used. As the gene derived from prokaryotes, the gene derived from a bacterium belonging to the genus Escherichia, Corynebacterium, Brevibacterium, Microbacterium, Bacillus, Staphylococcus, Streptococcus or Serratia and involved in the biosynthesis of the amino acid of the present invention or the metabolism relating to the biosynthesis is preferably used.

As the most preferable source of the gene, amino acid-producing strains and amino acid analog-resistant strains derived from the bacteria described above are used. The amino acid analog-resistance can be conferred on a plasmid after cloning.

As an example of the source of the gene involved in the biosynthesis of histidine, the chromosomal DNAs of Corynebacterium glutamicum C156 and Escherichia coli K12 ATCC 23740 are mentioned. C156 which is resistant to 1, 2,4-triazole-3-alanine and capable of producing histidine was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology under accession number FERM P-6910 on February 2, 1983. The deposit was transferred to the deposit under the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure under accession number FERM BP-453 on January 19, 1984.

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As an example of the source of the gene involved in the biosynthesis of tryptophan, the gene coding for anthranilic acid synthetase in the chromosomal DNA of Brevibacterium flavum ATCC 14067 or Corynebacterium glutamicum ATCC 13032, and the like are mentioned.

As the gene involved in the metabolism relating to the biosynthesis of phenylalanine, the genes coding for chorismate mutase and prephenate dehydratase are mentioned. Further, the above-mentioned genes on which the phenotype resistant to phenylalanine, tyrosine or an analog thereof such as para-fluorophenylalanine (PFP) is conferred are used. As the source of such a gene, Corynebacterium glutamicum K38 resistant to PFP is mentioned.

As an example of the source of the gene involved in the biosynthesis of isoleucine, a DNA fragment containing the gene coding for the enzyme involved in the biosynthesis of threonine from aspartic acid is mentioned.

As the enzyme involved in the biosynthesis of threonine from aspartic acid, aspartate kinase, aspartate-semialdehyde dehydrogenase, homoserine dehydrogenase, homoserine kinase and threonine synthase [Agr. Biol. Chem., 38, 993 (1974)] are mentioned.

As a preferable example of the gene coding for the enzyme involved in the biosynthesis of threonine, the recombinant plasmid pEthr1 containing the threonine operon DNA of Escherichia coli K-12 is used.

As the source of the gene involved in the biosynthesis of tyrosine of the present invention, Escherichia coli JA194 [Proc. Natl. Acad. Sci., 74, 487-491 (1977)] is preferably used.

Metabolic pathway and regulation systems of aromatic amino acids in microorganisms have been studied in detail on Escherichia coli, Bacillus subtilis, and glutamic acid-producing microorganisms such as strains of the genus of Corynebacterium and Brevibacterium [Agr. Chem. Soc. Japan, 50 (1), R79 - R87 (1976) and Ann. Rev. Biochemistry 47, 533 (1978)]. The gene involved in the biosynthesis of tyrosine of the present invention is a DNA carrying a genetic information of at least

one of enzymes involved directly or indirectly in the biosynthesis of these aromatic amino acids. The genes encoding for enzymes subjected to regulation on the biosynthetic pathway, i.e. 3-deoxy-D-arabino-heptulosonate 7-phosphate (referred to as DAHP hereinafter) synthetase (referred to as DAHPase hereinafter), chorismate mutase (referred to as CMase hereinafter), prephenate dehydrogenase (referred to as PDGase hereinafter) and pretyrosine aminotransferase are preferably used. Further, the genes of strains which are relieved from feed back inhibition and repression *a priori* or *a posteriori* are applicable.

As the enzyme involved in the biosynthesis of L-arginine, N-acetylglutamate synthetase, N-acetylglutamokinase, N-acetylglutamate-γ-semialdehyde dehydrogenase, N-acetylornithine-δ-aminotransferase, acetylornithine deacetylase, N-acetylglutamate-acetylornithine acetyltransferase, ornithine carbamoyltransferase, arginosuccinate synthetase, argininosuccinase, and the like [Agr. Biol. Chem., 43, 1899-1903 (1979)] are mentioned.

As the gene encoding for the enzymes involved in the biosynthesis of L-arginine, the DNA carrying the genetic information of at least one of these enzymes is used.

As the DNA containing the gene involved in the biosynthesis of arginine used in the present invention, genes located convergently at around 90 minutes on the chromosomal map of *Escherichia coli* K-12, containing the genes encoding for acetylornithine deacetylase (argE), N-acetylglutamate-γ-semialdehyde dehydrogenase (argC), N-acetylglutamokinase (argB) and argininosuccinase (argH) [Glansdorff, N.: Genetics, 51, 167 (1965)] are mentioned.

In the example, the recombinant plasmid pEarg1 containing the DNA of *Escherichia coli* K-12 genes involved in the biosynthesis of arginine is used.

pEarg1 can be obtained as a recombinant of pLC20-10 and pCE53 using a host-vector system of *Escherichia coli*. pLC20-10 is obtained from the gene bank of *Escherichia coli* K-12 and is known as a plasmid carrying the genes involved in the

biosynthesis of arginine described above [Clarke, L. et al.: Cell, 9, 91 (1976)].

The vector used in the present invention should autonomously replicate in cells of the host microorganism. Preferably, plasmids isolated from microorganisms belonging to the genus Corynebacterium by the present inventors or derivatives thereof such as pCG1 (Japanese Published Unexamined Patent Application No. 134500/82), pCG2 (Japanese Published Unexamined Patent Application No. 35197/83), pCG4 (Japanese Published Unexamined Patent Application No. 183799/82), pCE51, pCE52 (Japanese Published Unexamined Patent Application No. 126789/83), pCE53 (Japanese Published Unexamined Patent Application No. 25398/83), pCE54, pCG11, pCB101 (Japanese Published Unexamined Patent Application No. 105999/83), and the like are mentioned.

Microorganisms carrying these plasmids have been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, and the American Type Culture Collection, U.S.A. under the following accession numbers.

Plasmid	FERM P-	ATCC
pCG1	5865	31808
pCG2	5954	31832
pCG4	5939	31830
pCE54	–	39019
pCG11	–	39022
pCB101	–	39020

Plasmids pCE51, 52, 53 and 54 can be introduced from the plasmids mentioned above as follows.

For example, pCE51 is prepared as follows.

pCG1 is isolated from the cultured cells of Corynebacterium glutamicum 225-57 (FERM P-5865, ATCC 31808) by the method described in the specification of Japanese Published Unexamined Patent Application No. 134500/82. pGA22 is isolated from the cultured cells of Escherichia coli harboring the plasmid by a conventional method [An, G. et al.,: J.

Bacteriol., 140, 400 (1979)]. Plasmid pCG1 is linearized with restriction endonuclease BglII and a fragment of pGA22 digested with BamHI and containing kanamycin resistant (Km^R) gene is ligated to the linearized pCG1 using the same cohesive ends of both plasmids. Isolation of pCE51 from the ligated DNA mixture is achieved by selecting the transformants belonging to the genus Corynebacterium or Brevibacterium and containing Km^R derived from pGA22, and analyzing the plasmid in the transformant.

pCE51 has a molecular weight of about 6 Kb and cleavage sites for HincII, HindIII, SmaI, XhoI and EcoRI and gives Km^R phenotype.

pCE52 and pCE53 are prepared as follows.

Plasmid pCG1 is isolated from the cultured cells of Corynebacterium glutamicum 225-57 (FERM P-5865, ATCC 31808) by the method described in the above application and plasmid pGA22 is isolated from the cultured cells of Escherichia coli harboring the plasmid by a conventional method. pCG1 with a unique BglII site is linearized with restriction enzyme BglII and pGA22 with two BamHI sites are partially digested with BamHI. The cohesive ends of both plasmids are annealed and ligated with T4 phage DNA ligase to make a composite molecule. Selection of the recombinant plasmids from the ligation mixture is carried out by isolating transformants of the genus Corynebacterium or Brevibacterium on the basis of drug-resistances derived from pGA22 and then analyzing the plasmids in the transformants.

pCE52 and pCE53 have a molecular weight of about 10.9 Kb and cleavage sites for EcoRI, SalI, SmaI and XhoI. While pCE52 gives the phenotypes of resistance to chloramphenicol (Cm^R) and Km^R, pCE53 gives resistance to tetracycline (Tc^R), Cm^R and Km^R phenotypes. Since the cleavage site for XhoI is present in the Km^R gene, selection by insertional inactivation (prevention of the expression of a gene by the insertion of a DNA fragment into the gene) is possible.

Transformation with the ligated DNA mixture is carried out using protoplasts of the genus Corynebacterium or

Brevibacterium, and the method described in Japanese Published Unexamined Patent Application Nos. 186492/82 and 186489/82.

Among the genes responsible for drug resistance derived from pGA22, those except for the resistance gene which is insertionally inactivated are used for selection. In no DNA adding system, transformants are recovered as a colony regenerated on a hypertonic agar medium containing generally 0.4 - 1.6 μg/mℓ Tc, 2.5 - 5 μg/mℓ Cm, 100 - 800 μg/mℓ Km, 100 - 400 μg/mℓ Sm or 200 - 1,000 μg/mℓ Spec which does not allow the reversion to normal cells of the recipient protoplasts which are not treated with the ligation mixture. Alternatively, transformants are regenerated unselectively on a regeneration medium, and the resultant cells are scraped and resuspended, followed by the isolation of those cells grown on an agar medium containing a drug in a concentration wherein the recipient normal cells can not grow, that is, generally 0.5 - 4 μg/mℓ Tc, 2 - 15 μg/mℓ Cm, 2 - 25 μg/mℓ Km, 5 - 50 μg/mℓ Sm or 50 - 500 μg/mℓ Spec. Some of the transformants selected by Tc^R, Cm^R or Km^R are simultaneously endowed with other drug-resistances derived from plasmid pGA22.

Plasmid DNAs in these transformants can be isolated from cultured cells of the transformants and purified according to the methods described in Japanese Published Unexamined Patent Application Nos. 134500/82 and 186489/82. The structures of the DNAs can be determined by digesting them with various restriction endonucleases and analyzing the DNA fragments by agarose gel electrophoresis. The plasmids isolated from the transformants are pCE51, pCE52 and pCE53. Recovery of plasmids from the strains is carried out according to the methods described in Japanese Published Unexamined Patent Application Nos. 134500/82, 183799/82 and 35197/83. Preparation of a recombinant DNA of a vector DNA with a DNA fragment containing a gene is carried out by conventional *in vitro* recombinant DNA technology.

In vitro recombinant DNA technology is carried out by cleavage and ligation of a donor DNA containing a desired gene to a vector DNA (refer to Japanese Published Unexamined Patent

Application No. 126789/83, USP 4,237,224).

The ligase reaction gives recombinants containing genes other than the desired gene. The desired recombinant DNA can be obtained by directly transforming a microorganism of the genus Corynebacterium or Brevibacterium with the ligated DNA mixture, selecting the transformants having the phenotype derived from the desired gene and isolating the desired recombinant DNA from the cultured cells of the transformants. Instead of cloning the desired gene directly in a microorganism of the genus Corynebacterium or Brevibacterium, the desired gene can be cloned by using another host-vector system such as Escherichia coli. Then, it is recloned in vitro into a vector of the genus Corynebacterium or Brevibacterium to transform these microorganisms and transformants containing the desired recombinant plasmid are selected as mentioned above.

The method of cloning a gene in a host microorganism of Escherichia coli is described in, for example, Methods in Enzymology, 68, Ray Wu (Ed) Academic Press New York (1979).

The following references are helpful for the construction of recombinant DNA:

S.N. Cohen, et al., U.S.P. No. 4,237,224;

Idenshi Sosa Jikkenho, edited by Yasuyuki Takagi, printed by Kodansha Scientific (1980);

Methods in Enzymology 68, Recombinant DNA, edited by Ray Wu, Academic Press, 1979

Japanese Published Unexamined Patent Application No. 126789/83.

Microorganisms belonging to the genus Corynebacterium or Brevibacterium and which are competent for incorporating DNAs may be used as the host microorganisms in the present invention. The following are examples of a suitable host microorganism.

	Accession Number	
	FERM P-	ATCC
Corynebacterium glutamicum		13032
Coryn bacterium glutamicum L-15	5946	31834
Corynebacterium glutamicum LA-105		
Corynebacterium glutamicum K-38	7087	
Corynebacterium glutamicum K-43	7162	
Corynebacterium herculis		13868
Corynebacterium herculis L-103	5947	31866
Corynebacterium acetoacidophilum		13870
Corynebacterium lilium		15990
Brevibacterium divaricatum		14020
Brevibacterium divaricatum L-204	5948	31867
Brevibacterium lactofermentum		13869
Brevibacterium lactofermentum L-312	5949	31868
Brevibacterium flavum		14067
Brevibacterium immariophilium		14068
Brevibactrerium thiogenitalis		19240

Transformation of the host microorganisms with recombinant DNAs is carried out by the following steps:

1) Preparation of protoplasts from cultured cells;
2) Transformation of the protoplasts with a recombinant DNA;
3) Regeneration of the protoplasts to normal cells and selection of a transformant;

These steps are described in detail below.

1. Preparation of protoplasts from cultured cells:

The preparation of protoplasts is carried out by culturing a microorganism under conditions which render it sensitive to lysozyme, a lytic enzyme, and treating the cultured cells with lysozyme in a hypertonic solution to remove the cell wall. In order to render microbial cells sensitive to lysozyme, reagents inhibiting the synthesis of bacterial cell walls are used. For example, microbial cells sensitive to lysozyme are

obtained by adding, during the logarithmic growth phase, an amount of penicillin which does not inhibit or sub-inhibits the growth and then continuing culturing for several generations.

For culturing, any medium wherein the microorganism can grow may be used. For example, a nutrient medium NB (pH 7.2) consisting of 20 g/ℓ powdered bouillon and 5 g/ℓ yeast extract and a semi-synthetic medium SSM (pH 7.2) consisting of 10 g/ℓ glucose, 4 g/ℓ NH_4Cl, 2 g/ℓ urea, 1 g/ℓ yeast extract, 1 g/ℓ KH_2PO_4, 3 g/ℓ K_2HPO_4, 0.4 g/ℓ $MgCl_2 \cdot 6H_2O$, 10 mg/ℓ $FeSO_4 \cdot 7H_2O$, 0.2 mg/ℓ $MnSO_4 \cdot (4-6)H_2O$, 0.9 mg/ℓ $ZnSO_4 \cdot 7H_2O$, 0.4 mg/ℓ $CuSO_4 \cdot 5H_2O$, 0.09 mg/ℓ $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg/ℓ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 μg/ℓ biotin and 1 mg/ℓ thiamine hydrochloride are used.

Microbial cells are inoculated in the medium and culturing is carried out with shaking.

The optical density (OD) of the culture medium at 660 nm is monitored with a colorimeter and penicillin, such as penicillin G, is added to the medium at an initial stage of the logarithmic growth phase (OD : 0.1 - 0.4) in a concentration of 0.1 to 2.0 U/mℓ. Culturing is continued to an OD value of 0.3 - 0.5, and then cells are harvested and washed with the SSM medium. The washed cells are resuspended in a suitable hypertonic medium such as PFM medium (pH 7.0 - 8.5) wherein 0.4M sucrose and 0.01M $MgCl_2 \cdot 6H_2O$ are added to 2 fold diluted SSM medium, and RCG medium (pH 7.0 - 8.5) consisting of 5 g/ℓ glucose, 5 g/ℓ casein hydrolysate, 2.5 g/ℓ yeast extract, 3.5 g/ℓ K_2HPO_4, 1.5 g/ℓ KH_2PO_4, 0.41 g/ℓ $MgCl_2 \cdot 6H_2O$, 10 mg/ℓ $FeSO_4 \cdot 7H_2O$, 2 mg/ℓ $MnSO_4 \cdot (4-6)H_2O$, 0.9 mg/ℓ $ZnSO_4 \cdot 7H_2O$, 0.4 mg/ℓ $CuSO_4 \cdot 5H_2O$, 0.09 mg/ℓ $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg/ℓ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 μg/ℓ biotin, 2 mg/ℓ thiamine hydrochloride and 135 g/ℓ sodium succinate or RCGP medium which consists of RCG medium and 3% polyvinyl pyrrolidone. To the cell suspension, lysozyme is added to a final concentration of 0.2 to 10 mg/mℓ, and the mixture is allowed to react at a temperature of 30 to 37°C. Protoplast formation proceeds with time and is monitored with an optical microscope. The period required for the conversion of most cells to protoplasts depends on the concentrations of the penicillin used for the lysozyme-sensitization and the amount of lysozyme

used. The period is 3 - 24 hours under the conditions mentioned above.

Since protoplasts formed are destroyed under hypotonic conditions, the extent of the formation of protoplast is determined indirectly from the number of normal cells surviving under hypotonic conditions. Generally, the ratio of surviving normal cells are kept below 10^{-4} per lysozyme-treated normal cell.

The protoplasts as prepared above have colony-forming (regenerating) ability on a suitable hypertonic agar medium. As the agar medium, a nutrient medium, a semi-synthetic medium or a synthetic medium containing various amino acids, which contains 0.3 to 0.8M sodium succinate and 0.5 to 6% polyvinyl pyrrolidone with a molecular weight of 10,000 or 40,000 is preferably used. Generally, a semi-synthetic medium RCGP (pH 7.2) wherein 1.4% agar is added to the RCGP agar medium is used. Regeneration is carried out at a temperature of 25 to 35°C. The cultivation time required for the regeneration of protoplasts depends upon the strain used, and usually in 10 to 14 days colonies can be picked up. The efficiency of the regeneration of protoplasts on the RCGP medium also depends on the strain used, the concentrations of the penicillin added during the cultivation and the concentration of lysozyme used. The efficiency is generally 10^{-2} - 10^{-4} cells per normal cell treated with lysozyme.

2. Transformation of the protoplasts with a recombinant DNA:

Introduction of a recombinant DNA into the protoplast is carried out by mixing the protoplast and the DNA in a hypertonic solution which protects the protoplast and by adding to the mixture polyethyleneglycol (PEG, average molecular weight: 1,540 - 6,000) or polyvinylalcohol (PVA, degree of polymerization: 500 - 1,500) and a divalent metal cation which stimulates the uptake of DNA. As a stabilizing agent for the hypertonic conditions, those generally used to protect protoplasts of other microorganisms such as sucrose and sodium

succinate are also employed. PEG and PVA can be used at a final concentration of 5 to 60% and 1 to 20%, respectively. Divalent metal cations such as Ca^{++}, Mg^{++}, Mn^{++}, Ba^{++} and Sr^{++} are effectively used alone or in combination at a final concentration of 1 to 100 mM. Transformation is carried out satisfactorily at 0 to 25°C.

3. Regeneration of the protoplasts to normal cells and selection of a transformant:

Regeneration of the protoplast transformed with a recombinant DNA is carried out in the same way as mentioned above by spreading the protoplast on a hypertonic agar medium such as RCGP medium containing sodium succinate and polyvinyl pyrrolidone and incubating at a temperature wherein normal cells can grow, generally 25 to 35°C. Transformants are obtained by selecting the phenotypes derived from donor DNAs. The selection by characteristic phenotype endowed may be carried out simultaneously with regeneration on a hypertonic agar medium or may be carried out on a hypotonic agar medium after non-selective reversion to normal cells on a hypertonic agar medium.

In the case of using the lysozyme-sensitive strains described as the preferred host microorganisms, transformation may be carried out by the steps described in (1) to (3) except that the cultured cells are directly treated with lysozyme without prior treatment with penicillin in step (1). In that case, transformants are obtained at an efficiency of 10^{-2} to 10^{-4} per regenerated cell.

The following strains are the examples of the transformants obtained by the present invention.

Histidine-producing strains

Corynebacterium glutamicum K32, ATCC39281
Corynebacterium herculis K33, ATCC39282
Brevibacterium flavum K34, ATCC39283
Brevibacterium lactofermentum K35, ATCC39284
Corynebacterium glutamicum K49, FERM BP-464

Tryptophan-producing strains

Corynebacterium glutamicum K20, ATCC39035

Corynebacterium glutamicum K31, ATCC39280
Corynebacterium glutamicum K37, ATCC39285

Phenylalanine-producing strain
Corynebacterium glutamicum K39, FERM P-7088

Isoleucine-procucing strains
Corynebacterium glutamicum K41, FERM P-7161
Brevibacterium flavum K42, FERM BP-355

Tyrosine-producing strains
Corynebacterium glutamicum K44, FERM P-7163
Corynebacterium glutamicum K45, FERM P-7164

Arginine-producing strains
Corynebacterium glutamicum K46, FERM BP-356
Corynebacterium herculis K47, FERM BP-367
Brevibacterium flavum K48, FERM BP-357

The phenotypic expression of the recombinant DNA is carried out by growing the transformant in a conventional nutrient medium. Appropriate reagents may be added to the medium according to the phenotypes expected from the gene DNA or vector DNA on the recombinant DNA.

The thus obtained transformant is cultured in a conventional manner used in the production of amino acids by fermentation. That is, the transformant is cultured in a conventional medium containing carbon sources, nitrogen sources, inorganic materials, amino acids, vitamines, etc. under aerobic conditions, with adjustment of temperature and pH. Amino acids, thus accumulated in the medium are recovered.

As the carbon source, various carbohydrates such as glucose, glycerol, fructose, sucrose, maltose, mannose, starch, starch hydrolyzate and molasses, polyalcohols and various organic acids such as pyruvic acid, fumaric acid, lactic acid and acetic acid may be used. According to the assimilability of the microorganism strain used, hydrocarbon and alcohols are employed. Blackstrap molasses is most preferably used.

As the nitrogen source, ammonia, various inorganic or organic ammonium salts such as ammonium chloride, ammonium sulfate, ammonium carbonate and ammonium acetate, urea, and

nitrogenous organic substances such as peptone, NZ-amine, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, fish meal or its digested product, defatted soybean or its digested product and chrysalis hydrolyzate are appropriate.

As the inorganic materials, potassium dihydrogenphosphate, dipotassium hydrogenphosphate, ammonium sulfate, ammonium chloride, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate and calcium carbonate may be used. Vitamines and amino acids required for the growth of microorganisms may not be added, provided that they are supplied with other components mentioned above.

Culturing is carried out under aerobic conditions with shaking or aeration-agitation. Culturing temperature is preferably 20 to 40°C. The pH of the medium during culturing is maintained around neutral. Culturing is continued until a considerable amount of an amino acid is accumulated, generally for 1 to 5 days.

After completion of the culturing, cells are removed and the amino acid is recovered from the culture liquor by conventional manners such as treatment with active carbon or ion exchange resin.

In spite of the high similarity in microbiological characteristics, so called glutamic acid-producing microorganisms which produce glutamic acid in large amounts are classified into various species and even into different genera such as *Corynebacterium* and *Brevibacterium*, which is probably because of their industrial importance. However, it has been pointed out that these microorganisms should belong to one species because of nearly the same composition of amino acids in the cell wall and the base composition of DNAs. Recently, it has been reported that these microorganisms have at least 70 to 80% homology in DNA-DNA hybridization, indicating that these microorganisms are closely related. See, e.g., Komatsu, Y.: Report of the Fermentation Research Institute, No. 55, 1 (1980), and Suzuki, K., Kaneko, T., and Komagata, K.: Int. J. Syst. Bacteriol., 31, 131 (1981).

In consideration of the facts mentioned above, it is readily assumed that the usefulness of the present invention is applicable to all the glutamic acid-producing microorganisms. In order to stably maintain recombinant DNA molecules and express the DNA in these species, slight differences of such properties of the host microorganisms as homology in the DNA are negligible and it is sufficient for host microorganisms to allow the autonomous replication of plasmids and expression of genes on them. That these microorganisms have such abilities is apparent from the facts that plasmid pCG4 which was isolated from Corynebacterium glutamicum 225-250 (Japanese Published Unexamined Patent Application No. 183799/82) and having an streptomycin and/or spectinomycin resistant gene could replicate in the glutamic acid-producing microorganisms such as those belonging to the genus Corynebacterium or Brevibacterium and that the gene responsible for the resistance could be expressed (Japanese Published Unexamined Patent Application No. 186492/82). Further, as described in the production of amino acids of the present invention, it is apparent that the gene expressed in Corynebacterium glutamicum is expressible in broad host microorganisms of the genera Corynebacterium, Brevibacterium and the like. Therefore, all the glutamic acid-producing microorganisms including those belonging to the genus Corynebacterium or Brevibacterium as well as the species Corynebacterium glutamicum are competent as the host microorganism of the present invention.

For the strains appearing in the present specification, the accession numbers, deposition date and transfer date of the deposits under the Budapest Treaty of the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure are as follows.

	ATCC	FERM P (BP)	Deposition date (Transfer date) Year/Month/Day
Corynebacterium glutamicum L-15	31834		1981. 3. 9 (1984. 3.12)
Corynebacterium herculis L-103	31866		1981. 4. 3 (1984. 3.12)
Brevibacterium divaricatum L-204	31867		1981. 4. 3 (1984. 3.12)
Brevibacterium lactofermentum L-312	31868	(528)	1981. 4. 3 (1984. 4.20)
Corynebacterium glutamicum LA103/pCE54	39019		1981.12.11
Corynebacterium glutamicum LA103/pCB101	39020		1981.12.11
Corynebacterium glutamicum LA103/pEthr1	39021		1981.12.11
Corynebacterium glutamicum LA103/pCG11	39022		1981.12.11
Corynebacterium glutamicum K17	39032		1981.12.21
Corynebacterium glutamicum K18	39033		1981.12.21
Corynebacterium glutamicum K19	39034		1981.12.21
Corynebacterium glutamicum K20	39035		1981.12.21
Corynebacterium glutamicum K31	39280		1983. 1.28
Corynebacterium glutamicum K32	39281		1983. 1.28
Corynebacterium herculis K33	39282		1983. 1.28
Brevibacterium flavum K34	39283		1983. 1.28
Brevibacterium lactofermentum K35	39284		1983. 1.28

Corynebacterium glutamicum K37	39285		1983. 1.31
Corynebacterium glutamicum K36		6908 (451)	1983. 2. 8 (1984. 1.19)
Corynebacterium glutamicum H33		6909 (452)	1983. 2. 8 (1984. 1.19)
Corynebacterium glutamicum C156		6910 (453)	1983. 2. 8 (1984. 1.19)
Corynebacterium glutamicum K38		7087 (454)	1983. 5.19 (1984. 1.19)
Corynebacterium glutamicum K39		7088 (459)	1983. 5.19 (1984. 1.21)
Corynebacterium glutamicum K40		7160 (455)	1983. 7.21 (1984. 1.19)
Corynebacterium glutamicum K41		7161 (456)	1983. 7.21 (1984. 1.19)
Corynebacterium glutamicum K43		7162 (457)	1983. 7.21 (1984. 1.19)
Corynebacterium glutamicum K44		7163 (458)	1983. 7.21 (1984. 1.19)
Corynebacterium glutamicum K45		7164 (460)	1983. 7.21 (1984. 1.21)
Brevibacterium flavum K42		(355)	1983. 9.12
Corynebacterium glutamicum K46		(356)	1983. 9.12
Brevibacterium flavum K48		(357)	1983. 9.12
Corynebacterium herculis K47		(367)	1983. 9.21
Corynebacterium glutamicum K49		(464)	1984. 1.25

Brief Description of The Drawings

Fig. 1 illustrates the cleavage pattern with restriction endonucleases of pGH2.

Fig. 2 illustrates the process for construction of and the cleavage map for restriction endonucleases of pEthr1 wherein "Bgl II/Dam HI" with a broken line indicates a recombination site at the same cohesive ends formed by cleavage with both restriction endonucleases. The restriction endonucleases used in the preparation of the cleavage map are PstI, EcoRI and XhoI. Molecular weight of the plasmid is indicated as Kilobase pairs (Kb).

Fig. 3 illustrates the cleavage pattern with r striction endonucleases for plasmid pEaroF-1.

Fig. 4 illustrates the cleavage pattern with restriction endonucleases for plasmid pKmlaroF1.

In Figs. 3 and 4, the horizontal arrows show orientation of transcription of genes.

Fig. 5 illustrates the process for construction of plasmid pEarg1. The restriction endonuclceases used in the preparation of the cleavage map are PstI, BamHI and SalI.

Example 1

Cloning of a gene involved in the biosynthesis of L-histidine derived from *Corynebacterium glutamicum* C156 strain and production of L-histidine by the expression of the gene in *Corynebacterium glutamicum*, *Corynebacterium herculis*, *Brevibacterium flavum* and *Brevibacterium lactofermentum*:

1) Preparation of the chromosomal DNA of *Corynebacterium glutamicum* C156 and plasmid pCG11:

The chromosomal DNA was prepared from *Corynebacterium glutamicum* C156 (FERM P-6910, FERM BP-453) which is resistant to 1,2,4-triazole-3-alanine and capable of producing histidine as follows:

A seed culture was inoculated into 400 mℓ of a semi-synthetic medium SSM. NB medium was used for seed culture. Culturing was carried out with shaking at 30°C. The optical density (OD) at 660 nm was monitored with a Tokyo Koden colorimeter and penicillin G was added at an OD value of 0.2 to a concentration of 0.5 unit/mℓ. Culturing was continued to an

OD value of about 0.6.

Cells were harvested from the culture broth and washed with TES buffer (pH 8.0) consisting of 0.03M tris-(hydroxymethyl)aminomethane-HCl (referred to as Tris hereinafter), 0.005M EDTA and 0.05M NaCl. The cells were suspended in a lysozyme solution (pH 8.0) consisting of 12.5% sucrose, 0.1M NaCl, 0.05M Tris and 0.8 mg/mℓ lysozyme to make 10 mℓ of a suspension which was allowed to react at 37°C for 4 hours. High molecular chromosomal DNAs were isolated from the cells by the method of Saito _et al_., Biochim. Biophys. Acta, _72_, 619 (1963).

Separately pCG11 used as a vector plasmid was prepared from _Corynebacterium glutamicum_ LA 103/pCG11, ATCC 39022 which is a derivative of _Corynebacterium glutamicum_ L-22 and harbors pCG11 as follows.

The strain was grown with shaking at 30°C in 400 mℓ of NB medium to an OD value of about 0.7. Cells were harvested and washed with TES buffer. The cells were suspended in 10 mℓ of the aforementioned lysozyme solution and allowed to react at 37°C for 2 hours. Then 2.4 mℓ of 5M NaCl, 0.6 mℓ of 0.5M EDTA (pH 8.5) and 4.4 mℓ of a solution consisting of 4% sodium lauryl sulfate and 0.7M NaCl were added successively. The mixture was stirred slowly and allowed to stand on an ice water bath for 15 hours.

The whole lysate was centrifuged at 4°C under 69,400 x g for 60 minutes. The supernatant fluid was recovered and 10% (by weight) polyethyleneglycol (PEG) 6,000 (product of Nakarai Kagaku Yakuhin Co.) was added. The mixture was stirred slowly to dissolve completely and then kept on an ice water bath. After 10 hours, the mixture was subjected to centrifugation at 1,500 x g for 10 minutes to recover a pellet. The pellet was redissolved gently in 5 mℓ of TES buffer and 2.0 mℓ of 1.5 mg/mℓ ethidium bromide was added. Then, cesium chloride was added to adjust the density of the mixture to 1.580. The solution was centrifuged at 18°C at 105,000 x g for 48 hours. After the density gradient centrifugation, a covalently-closed circular DNA was detected under UV irradiation as a high density band

located in the lower part of the centrifugation tube. The band was taken out from the side of the tube with an injector to obtain a fraction containing pCG11 DNA. To remove ethidium bromide, the fraction was treated five times with an equal amount of cesium chloride saturated isopropyl alcohol solution consisting of 90% by volume isopropyl alcohol and 10% TES buffer solution. Then, the residue was dialysed against TES buffer solution to obtain pCG11 plasmid DNA.

2) Cloning of the gene involved in the biosynthesis of histidine in Corynebacterium glutamicum C156:

In this step, 10 units of restriction enzyme BglII (product of Takara Shuzo Co. the restriction enzymes hereinafter are all products of Takara Shuzo Co., unless otherwise specified) was added to 200 μℓ of a BglII reaction solution (pH 7.5) consisting of 10 mM Tris (pH 7.5), 7 mM $MgCl_2$, 60 mM NaCl and 7 mM 2-mercaptoethanol and containing 3 μg of pCG11 plasmid DNA and 9 μg of the chromosomal DNA prepared as above. The mixture was allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction. 40 μℓ of a T4 ligase buffer solution I (pH 7.6) consisting of 200 mM Tris, 66 mM $MgCl_2$ and 100 mM dithiothreitol, 40 μℓ of 5 mM ATP solution, 0.3 μℓ of T4 ligase (product of Takara Shuzo Co., 1 unit/μℓ, the same shall apply hereinafter) and 120 μℓ of H_2O were added. The mixture was allowed to react at 12°C for 16 hours and then used to transform Corynebacterium glutamicum LH33 which requires histidine and is sensitive to lysozyme.

The transformation was carried out using the protoplast of LH33 strain. The seed culture of LH33 was inoculated into NB medium and culturing was carried out with shaking at 30°C. Cells were harvested at an OD value of 0.6. The cells were suspended at about 10^9 cells/mℓ in RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and stirred slowly at 30°C for 5 hours to obtain protoplasts.

Then, 0.5 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 5 minutes. The

protoplasts were resuspended in 1 mℓ of TSMC buffer (pH 7.5) consisting of 10 mM magnesium chloride, 30 mM calcium chloride, 50 mM Tris and 400 mM sucrose and again subjected to centrifugation and washing. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. 100 μℓ of a mixture (1 : 1 by volume) of a two-fold concentrated TSMC buffer and the ligated DNA mixture described above was added to the protoplast suspension. Then, 0.8 mℓ of a solution containing 20% PEG 6,000 in TSMC buffer solution was added to the mixture. After 3 minutes, 2 mℓ of RCGP medium (pH 7.2) was added and the mixture was centrifuged at 2,500 x g for five minutes. The supernatant fluid was removed and the protoplasts were suspended in 1 mℓ of RCGP medium. Then, 0.2 mℓ of the suspension was spread on RCGP agar medium (pH 7.2) containing 400 μg/mℓ spectinomycin and 1.4% agar and incubated at 30°C for 7 days.

All the colonies formed on the agar medium were scraped, washed with physiological saline solution and centrifuged two times. The cells were spread on a minimal agar medium M1 (pH 7.2) consisting of 10 g/ℓ glucose, 1 g/ℓ $NH_4H_2PO_4$, 0.2 g/ℓ KCl, 0.2 g/ℓ $MgSO_4 \cdot 7H_2O$, 10 mg/ℓ $FeSO_4 \cdot 7H_2O$, 0.2 mg/ℓ $MnSO_4 \cdot (4\text{-}6)H_2O$, 0.9 mg/ℓ $ZnSO_4 \cdot 7H_2O$, 0.4 mg/ℓ $CuSO_4 \cdot 5H_2O$, 0.09 mg/ℓ $Na_2B_4O_7 \cdot 10H_2O$, 0.04 mg/ℓ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 μg/ℓ biotin, 2.5 mg/ℓ p-aminobenzoic acid, 1 mg/ℓ thiamine hydrochloride and 16 g/ℓ agar and containing 100 μg/mℓ spectinomycin and incubated at 30°C for 2 days. The transformants which are resistant to spectinomycin and do not require histidine were obtained from the colonies formed.

A plasmid DNA was isolated from cells of one of the transformants by the same ethidium bromide-cesium chloride density gradient centrifugation method as in step (1) above. The plasmid DNA was digested with restriction endonucleases alone or in combination and analyzed by agarose gel electrophoresis to determine the cleavage pattern of the plasmid DNA. The plasmid was named pPH8. pPH8 has the structure wherein about 10.6 Kb of a DNA fragment is inserted into the BglII site of pCG11.

H33 strain which is the parent of [illegible]33 strain (FERM P-6909, FERM BP-452) requiring histidine and resistant to lysozyme was retransformed with pPH8 DNA. All of the transformants selected for spectinomycin-resistance simultaneously became histidine prototroph.

Therefore, it is certain that a gene involved in the biosynthesis of histidine in histidine-producing Corynebacterium glutamicum C156 was cloned in the plasmid.

The cloning of the gene involved in the biosynthesis of histidine can also be carried out using H33 strain as a host from the beginning.

3) Production of L-histidine by Corynebacterium glutamicum carrying pPH8:

Corynebacterium glutamicum LA-103 was transformed with pPH8 DNA and a spectinomycin-resistant transformant was selected on RCGP agar medium containing 400 μg/mℓ spectinomycin. The transformant could grow on a minimal agar medium containing 3 mg/mℓ 2-thiazole alanine. After the transformant was purified, the plasmid was isolated and its structure was analysed as mentioned above to confirm that the plasmid has the same structure as that of pPH8. A strain having pPH8, Corynebacterium glutamicum LA103/pPH8 has been deposited with the American Type Culture Collection, U.S.A. as Corynebacterium glutamicum K32 ATCC 39281.

Corynebacterium glutamicum LA103/pCG11 (ATCC 39022) and LA103/pPH8 (ATCC 39281) were tested for L-histidine production as follows.

A loopful of cells cultured in NB agar medium at 30°C overnight was inoculated in 5 ml of a production medium P5 adjusted to pH 7.4 with ammonia consisting of 12% molasses as sugar, 0.2% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.25% NaCl, 2.3% $(NH_4)_2SO_4$, 0.2% urea and 2% $CaCO_3$, and containing 200 μg/mℓ each arginine and methione. Culturing was carried out at 30°C for 75 hours. The amount of L-histidine formed was determined by a colorimetric method using sulfanilic acid (Pauly) reagent [H. Pauly, Hoppe-Seylers; Z. Physiolo. Chem., 42, 508 (1904),

ibid, 94, 284 (1915)]. The results are shown in Table 1.

Table 1

Strain	Amount of L-histidine (mg/mℓ)
LA103/pCG11	0
LA103/pPH8 (K32)	2.6

4) Production of L-histidine by pPH8 containing-strains, *Corynebacterium herculis*, *Brevibacterium flavum* and *Brevibacterium lactofermentum*:

In order to introduce plasmid pPH8 into *Corynebacterium herculis* ATCC 13868, *Brevibacterium flavum* ATCC 14067 and *Brevibacterium lactofermentum* ATCC 13869, these strains were transformed as follows.

Each strain was grown in SSM medium and penicillin G was added at an OD_{660} value of 0.2 to a concentration of 0.3 unit/mℓ. Culturing was continued to an OD_{660} value of 0.6. Cells were harvested and converted to protoplasts in RCGP medium containing 1 mg/mℓ lysozyme as mentioned above. Transformation was carried out using pPH8 as mentioned above and colonies formed on RCGP agar medium containing 400 μg/mℓ spectinomycin were selected as transformants.

Plasmid DNAs were prepared from the cells of the purified spectinomycin-resistant transformants according to the method described in Japanese Published Unexamined Patent Application Nos. 183799/82 and 134500/82. It was confirmed from the cleavage pattern that these plasmid DNAs have the same structure as that of pPH8. Therefore, it is certain that plasmid pPH8 which is a derivative of plasmid pCG11 is autonomously replicable in *Corynebacterium herculis*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* and that plasmid pCG11 is broadly useful among these bacteria.

pPH8 containing-strains, *Corynebacterium herculis* K33, *Brevibacterium flavum* K34 and *Brevibacterium lactofermentum* K35 have been deposited with the American Type Culture Collection

under ATCC 39282, 39283 and 39284.

These strains were tested for L-histidine production as follows.

One loopful of cells of each of pPH8 containing-strains as well as parent strains thereof cultured in NB agar medium at 30°C overnight was inoculated in 5 mℓ of a production medium P5. Culturing was carried out with shaking at 30°C for 75 hours. The amount of L-histidine formed was determined by the colorimetric method of Pauly. The results are shown in Table 2.

Table 2

Strain	Amount of L-histidine (mg/mℓ)
ATCC 13868	0
ATCC 13868/pPH8 (K33, ATCC 39282)	2.4
ATCC 14067	0
ATCC 14067/pPH8 (K34, ATCC 39283)	3.0
ATCC 13869	0
ATCC 13869/pPH8 (K35, ATCC 39284)	2.0

As is apparent from the above results, a gene involved in the biosynthesis of histidine derived from Corynebacterium glutamicum is expressed and contribute to the production of histidine in Corynebacterium herculis, Brevibacterium flavum and Brevibacterium lactofermentum.

Example 2

Construction of the recombinant plasmid containing a gene involved in L-histidine biosynthesis of Escherichia coli K-12 ATCC 23740, conferring 1,2,4-triazole-3-alanine-resistance on the plasmid by mutation and the production of L-histidine by a strain of Corynebacterium glutamicum harbouring the resistant plasmid:

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1) Preparation of chromosomal DNA of <u>Escherichia</u> <u>coli</u> K-12 ATCC 23740 and plasmid pCE53:

The chromosomal DNA was prepared from a histidine prototrophic strain of <u>Escherichia</u> <u>coli</u> K-12 ATCC 23740 by the phenol method of Smith (Smith M.G.: Methods in Enzymology <u>12</u> Part A., 545, 1967).

The vector plasmid, pCE53 was isolated according to the method of An (An. G., et al. J. Bacteriol. <u>140</u> 400, 1979) from <u>Escherichia</u> <u>coli</u> K-12 MM294/pCE53.

MM294/pCE53 was constructed by the following process. It was constructed by transforming <u>Escherichia</u> <u>coli</u> K12 MM294 (Muller-Hill, B. <u>et</u> <u>al</u>., in Protein Ligand Interactions, eds. Sund, H. and Blauer, G. p211, 1975) with the plasmid isolated from the cultured cells of <u>Corynebacterium</u> <u>glutamicum</u> LA103/pCE53 according to the method described above. The preparation of competent cells of MM294 were done as described in the method of Dagert [Dagert, M. <u>et</u> <u>al</u>., Gene <u>6</u>, 23 (1979)]. Kanamycin resistant transformants were selected on L agar medium (10 g/ℓ Bacto-trypton, 5 g/ℓ yeast extract, 5 μ/ℓ NaCl, 1 g/ℓ glucose and 16 g/ℓ agar, pH adjusted to 7.2) containing 25 μg/mℓ kanamycin. Plasmid DNA was isolated from one of the transformants by the method of An et al. The structure of the plasmid was analyzed by digesting with restriction endonucleases and was found to be the same as that of pCE53.

<u>Corynebacterium</u> <u>glutamicum</u> LA103/pCE53 was constructed as follows. The BglII-restricted pCG1 and BamHI partially-digested pGA22 were ligated with T4 ligase (product of Takara Shuzo Co.). <u>Corynebacterium</u> <u>glutamicum</u> LA103 was transformed with the ligation mixture according to the method described in Japanese Published Unexamined Patent Application Nos. 186492/82 and 186489/82. Kanamycin-resistant transformants were selected on RCGP agar medium containing 100 μg/mℓ kanamycin. Plasmids were isolated from these transformants and their structures were analyzed by cleavage with restriction endonucleases. A plasmid from one of the transformants contains pCG1 inserted in the BamHI site near the kanamycin-resistant gene of pGA22. The plasmid and the transformant containing it were designated as

pCE53 and LA103/pCE53, respectively.

2) Cloning of a gene involved in histidine biosynthesis from Escherichia coli ATCC 23740:

To 200 μℓ of a restriction enzyme PstI buffer [20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 0.01% bovine serum albumin, the same shall apply hereinafter] containing 3 μg of pCE53 plasmid DNA and 9 μg of the chromosomal DNA of Escherichia coli prepared as described above, 10 units of PstI was added. The mixture was incubated at 37°C for 60 min and then the reaction was stopped by heating at 65°C for 10 min. To the digestion mixture, 40 μℓ of T4 ligase buffer I was added together with 40 μℓ of 5 mM ATP, 0.3 unit of T4 ligase and 120 μℓ of water and the mixture was incubated at 12°C for 16 hr. The ligation mixture was used to transform Escherichia coli K-12 JC411 (hisG, leuB, argG, metB; Clark et al., Molec. Gen. Genet. 105, 1, 1969). The competent cells of JC411 were prepared according to the method of Dagert et al (Gene 6, 23, 1979). The cells were grown in 50 mℓ of L-broth [10 g/ℓ Bacto-trypton, 5 g/ℓ Yeast extract and 5 g/ℓ NaCl (pH 7.2), referred to as LB hereinafter] to the middle of the logarithmic phase of growth at 37°C with shaking. After the culture was kept in ice for 10 min, cells were harvested by centrifugation and suspended in 20 mℓ of cold 0.1M $CaCl_2$. The suspension was kept at 0°C for 20 min and then cells were harvested by centrifugation and suspended in 0.5 mℓ of cold 0.1M $CaCl_2$ and kept at 0°C for 18 hr.

50 μℓ of the ligation mixture was added to 150 μℓ of the $CaCl_2$-treated cell suspension. After the mixture was kept at 0°C for 10 min, it was incubated at 37°C for 3 min. Two mℓ of L medium was added to the mixture and cells were grown at 37°C for 2 hrs with shaking. After washing with a saline twice by centrifugation, cells were plated and grown on A agar medium Na_2HPO_4 8g, KH_2PO_4 2g, $(NH_4)_2SO_4$ 1g, $MgSO_4 \cdot 7H_2O$ 0.1g, thiamine·HCl 4 mg glucose 10g and agar 16g in 1 liter water, pH adjusted to 7.2, the same shall apply hereinafter) containing 50 μg each of leucine, arginine and methionine and 25 μg of kanamycin per mℓ at 37°C for 3 days. Plasmid DNAs were isolated

from the transformants as described above and their structures were analyzed by digesting with restriction endonucleases and agarose gel electrophoresis. A plasmid from one of the transformants, designated as pEG7, has about 4.8 Kb PstI DNA fragment in the unique PstI site of pCE53.

pEG7 DNA was used to retransform the competent cells of JC411 prepared as described above. All the transformants grown on NB agar medium containing 25 μg/mℓ kanamycin were found histidine prototrophs. Thus it is clear that plasmid pEG7 can complement hisG mutation of JC411.

3) Introduction of pEG7 into Corynebacterium glutamicum

Corynebacterium glutamicum LH73 (histidine requiring and lysozyme-sensitive) was transformed with pEG7 plasmid DNA by the protoplast transformation method described in Example 1(2). Km^r transformants grown on RCGP agar medium containing 200 μg/mℓ kanamycin had a plasmid having the same structure as the plasmid pEG7 as judged from the digestion pattern with restriction endonucleases. All the kanamycin-resistant transformants were found histidine prototrophs. It is certain that a gene involved in the biosynthesis of histidine derived from Escherichia coli expresses in Corynebacterium glutamicum.

4) Conferring resistance to a histidine analogue, 1,2,4 triazole-3-alanine into plasmid pEG7:

Corynebacterium glutamicum LA103/pEG7 constructed above was mutagenized with nitroso guanidine by a conventional method and plated on minimal agar medium M1 containing 1 mg/mℓ 1,2,4-triazole-3-alanine (referred to as TRA hereinafter). After incubation at 30°C for 5 days, colonies formed were scraped and suspended in a saline. The cell suspension was inoculated into NB medium containing 25 μg/mℓ kanamycin at a cell density of 10^7/mℓ and incubated at 30°C overnight with shaking. Plasmids were isolated from the cultured cells as described above (Example 1(1)). The mixture of plasmids isolated were used to re-transform Corynebacterium glutamicum LH73. A plasmid was isolated from one of Km^r transformants

which also acquired TRA-resistance. The plasmid has the same digestion pattern with restriction endonucleases as that of pEG7. It was designated pEG7t180.

5) Subcloning of the 4.8 Kb PstI DNA fragment of pEG7t180:

To confirm the presence of TRA^r mutation on the 4.8 Kb PstI DNA fragment derived from Escherichia coli, it was subcloned as follows. Each 3 μg of pEG7t180 and pCG11 DNAs were digested with PstI and ligated with T4 ligase. The ligation mixture was used to transform Corynebacterium glutamicum LA103 as described in Example 1(2). Spectinomycin-resistant transformants obtained were plated on A agar medium containing 1 mg/mℓ TRA, 50 μg/mℓ each arginine and methionine, NB agar medium containing 25 μg/mℓ kanamycin and NB medium containing 100 μg/mℓ spectinomycin. After incubation at 30°C for 5 days, a strain which was spectinomycin- and TRA-resistant and Km-sensitive was selected and purified. A plasmid was isolated from the cultured cells of the strain and analyzed by digestion with restriction endonucleases. The plasmid contains the 4.8 Kb PstI DNA fragment inserted at the unique PstI site of pCG11. It was designated as pCSt180.

LH73 was retransformed using pCSt180 DNA. Spectinomycin-resistant transformants were all histidine prototrophs.

Production tests of Corynebacterium glutamicum LA103 pEG7, LA103/pEG7t180, LA1031/pCG11 (ATCC39022) and LA103/pCSt180 (K-49) were done using production medium P5 supplemented with 200 μg/mℓ each arginine and methionine. Histidine accumulated after cultivation at 30°C for 75 hrs was measured colorimetrically as described in Example 1(3). The result is shown in Table 3.

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TABLE 3

Strain	Amount of L-histidine (mg/mℓ)
LA103/pEG7	0
LA103/pEG7t180	1.8
LA103/pCG11	0
LA103/pCSt180	2.0

These results indicate that the TRA^r mutation resides on the 4.8 Kb PstI DNA fragment derived from the chromosomal DNA of Escherichia coli, harboring a gene involved in the histidine biosynthesis and that the production of histidine by Corynebacterium glutamicum was due to the expression of the gene(s) on the fragment.

LA103/pCSt180 has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, under the accession number FERM BP-464, as Corynebacterium glutamicum K49.

Example 3

Cloning of the anthranilic acid synthetase gene of Brevibacterium flavum ATCC 14067 and production of tryptophan in Corynebacterium glutamicum:

1) Preparation of chromosomal DNA and plasmid pCE53:

The chromosomal DNA of Brevibacterium flavum ATCC 14067 was prepared as follows:

A seed culture was inoculated into 400 mℓ of a semi-synthetic medium SSM. NB medium was used for seed culture. Culturing was carried out with shaking at 30°C. The optical density (OD) at 660 nm was monitored with a Tokyo Koden colorimeter and penicillin G was added at an OD value of 0.2 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of about 0.6.

Cells were harvested from the culture broth and washed with TES buffer. The cells were suspended in a lysozyme solution (pH 8.0) consisting of 25% sucrose, 0.1M NaCl, 0.05M Tris and 0.8 mg/mℓ lysozyme (the same lysozyme solution was used hereinafter) to make 10 mℓ of a suspension which was allowed to react at 37°C for 4 hours. High molecular chromosomal DNAs were isolated from the cells by the method of Saito *et al*.

pCE53 used as a vector plasmid was isolated from *Corynebacterium glutamicum* L-22 harboring pCE53 as follows.

The strain was grown with shaking at 30°C in 400 mℓ of NB medium to an OD value of about 0.7. Cells were harvested and washed with TES buffer. The cells were suspended in 10 mℓ of the aforementioned lysozyme solution and allowed to react at 37°C for 2 hours. Then 2.4 mℓ of 5M NaCl, 0.6 mℓ of 0.5M EDTA (pH 8.5) and 4.4 mℓ of a solution consisting of 4% sodium lauryl sulfate and 0.7M NaCl were added successively. The mixture was stirred slowly and allowed to stand in an ice water bath for 15 hours.

The whole lysate was centrifuged at 4°C at 69,400 x g for 60 minutes. The supernatant fluid was recovered and 10% (by weight) polyethyleneglycol (PEG) 6,000 (product of Nakarai Kagaku Yakuhin Co.) was added. The mixture was stirred slowly to dissolve completely and then kept in an ice water bath. After 10 hours, the mixture was centrifuged at 1,500 x g for 10 minutes to recover a pellet. After the pellet was redissolved gently in 5 mℓ of TES buffer, 2.0 mℓ of 1.5 mg/mℓ ethidium bromide was added. Then, cesium chloride was added calmly to adjust the density of the mixture to 1.580. The solution was centrifuged at 18°C at 105,000 x g for 48 hours. After the density gradient centrifugation, a covalently-closed circular DNA was detected under UV irradiation as a high density band located in the lower part of the centrifugation tube. The band was taken out from the side of the tube with an injector to obtain a fraction containing pCE53 DNA.

To remove ethidium bromide, the fraction was treated five times with an equal amount of cesium chloride saturated isopropyl alcohol solution consisting of 90% by volume isopropyl

alcohol and 10% TES buffer solution. Then, the residue was dialysed against TES buffer solution.

pCE53 is a recombinant plasmid wherein plasmid pCG1 (Japanese Published Unexamined Patent Application No. 134500/82) of Corynebacterium glutamicum is combined with plasmid pGA22 of Escherichia coli described by An, G. et al., J. Bacteriol 140, 400 (1979). More specifically, pCE53 was constructed by inserting BglII restricted pCG1 into the BamHI site without the tetracycline-resistance gene of pGA22 and ligating by taking advantage of the same cohesive ends formed by both restriction enzymes. pCE53 has selective markers such as kanamycin-resistance derived from pGA22 and has only one cleavage site for SalI.

2) Cloning of the anthranilic acid synthetase gene:

10 units of restriction enzyme SalI was added to 200 μℓ of the SalI reaction solution containing 3 μg of pCE53 plasmid DNA and 9 μg of the chromosomal DNA prepared as above. The mixture was allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction. Then, 40 μℓ of the T4 ligase buffer II (pH 7.6) consisting of 660 mM Tris, 66 mM $MgCl_2$ and 100 mM dithiothreitol, 40 μℓ of 5 mM ATP, 0.4 μℓ of T4 ligase and 120 μℓ of H_2O were added to the digest. The mixture was allowed to react at 12°C for 16 hours.

3) Transformation with the recombinant plasmid:

The ligation mixture was provided for transformation as the recipient for transformation, LA 105, which is a mutant requiring anthranilic acid due to the lack of the anthranilic acid synthetase gene and derived from Corynebacterium glutamicum L-22, was used. The mutant was obtained by a conventional mutagenesis as a strain which could not grow on M1 agar medium and could grow on M1 agar medium containing 30 μg/mℓ anthranilic acid. Preparation of the protoplasts of LA 105 and transformation of the protoplasts were carried out using LA 105 cells grown on NB medium containing 100 μg/mℓ anthranilic acid as follows.

The seed culture of LA 105 strain was inoculated into NB medium and culturing was carried out with shaking at 30°C. Cells were harvested at an OD value of 0.6. The cells were suspended at about 10^9 cells/mℓ in RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and shaken slowly at 30°C for 5 hours to obtain protoplasts.

Then, 0.5 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 5 minutes. The protoplasts were resuspended in 1 mℓ of TSMC buffer and again subjected to centrifugation and washing. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. 100 μℓ of a mixture (1 : 1 by volume) of two-fold concentrated TSMC buffer solution and the ligated DNA mixture described above was added to the protoplast suspension. Then, 0.8 mℓ of TSMC buffer containing 20% PEG 6,000 was added to the mixture. After 3 minutes, 2 mℓ of RCGP medium (pH 7.2) was added and the mixture was centrifuged at 2,500 x g for 5 minutes. The supernatant fluid was removed and the protoplasts were suspended in 1 mℓ of RCGP medium. Then, 0.2 mℓ of the suspension was spread on RCGP agar medium (pH 7.2) containing 300 μg/mℓ kanamycin and 1.4% agar and incubated at 30°C for 7 days.

Colonies resistant to kanamycin grown on the selection plate were scraped, washed with physiological saline solution and centrifuged two times. The cells were spread on M1 minimal agar medium containing 20 μg/mℓ kanamycin and incubated at 30°C for 2 days. Transformants which were resistant to kanamycin and did not require anthranilic acid were selected.

Plasmid DNAs were insolated from the cells of these transformants in the same way as mentioned above. The plasmid pTrp 2 - 3, recovered from one of the transformants, was analyzed by digestion with various restriction endonucleases and agarose gel electrophoresis. As the result, the plasmid pTrp 2 - 3 was found to contain an about 7.1 Kb SalI DNA fragment inserted into the unique SalI site of pCE53.

Strain LA 105 was retransformed with pTrp 2 - 3 in the same way as mentioned above. The colonies grown on RCGP agar

medium containing 100 μg/mℓ tryptophan and 400 μg/mℓ kanamycin did not require anthranilic acid for growth and they had the same plasmid as pTrp 2 - 3 characterized by the cleavage pattern by SalI.

The result shows that the gene coding for anthranilic acid synthetase of Brevibacterium flavum ATCC 14067 is present in the cloned SalI DNA fragment of about 7.1 Kb and expressed in Corynebacterium glutamicum LA 105.

A microorganism containing pTrp 2 - 3, Corynebacterium glutamicum K20, has been deposited with the American Type Culture Collection, U.S.A. under accession number ATCC 39035.

4) Production of tryptopan by a transformant:

Plasmid pTrp 4 - 3 carrying the anthranilic acid synthetase gene of Brevibacterium flavum ATCC 14067 was obtained using plasmid pCE52 by the same method as mentioned above.

pCE52 is a recombinant plasmid wherein plasmid pCG1 (Japanese Published Unexamined Patent Application No. 134500/82) of Corynebacterium glutamicum is combined with plasmid pGA22 of Escherichia coli described by An, G. et al., J. Bacteriol 140, 400 (1979). It was constructed by inserting BglII restricted pCG1 into the BamHI site in the tetracycline-resistance gene of pGA22 and ligating by taking advantage of the same cohesive ends formed by both restriction enzymes. pCE52 has selective markers such as kanamycin resistance derived from pGA22 and has only one cleavage site for SalI.

pCE52 was isolated from the cells of Corynebacterium glutamicum L-22 containing pCE52 as follows:

The strain was grown with shaking at 30°C in 400 mℓ of NB medium to an OD value of about 0.7. Cells were harvested and washed with TES buffer. The cells were suspended in 10 mℓ of the lysozyme solution and allowed to react at 37°C for 2 hours. Then 2.4 mℓ of 5M NaCl, 0.6 mℓ of 0.5M EDTA (pH 8.5) and 4.4 mℓ of a solution consisting of 4% sodium lauryl sulfate and 0.7M NaCl were added successively. The mixture was stirred slowly and allowed to stand in an ice water bath for 15 hours.

The whole lysate was centrifuged at 4°C at 69,400 x g for 60 minutes. The supernatant fluid was recovered and 10% (by weight) polyethyleneglycol (PEG) 6,000 (product of Nakarai Kagaku Yakuhin Co.) was added. The mixture was stirred slowly to dissolve completely and then kept in an ice water bath. After 10 hours, the mixture was centrifuged at 1,500 x g for 10 minutes to recover a pellet. After the pellet was redissolved gently in 5 mℓ of TES buffer, 2.0 mℓ of 1.5 mg/mℓ ethidium bromide was added. Then, cesium-chloride was added to adjust the density of the mixture to 1.580. The solution was centrifuged at 18°C at 105,000 x g for 48 hours. After the density gradient centrifugation, a covalently-closed circular DNA was detected under UV irradiation as a high density band located in the lower part of the centrifugation tube. The band was taken out from the side of the tube with an injector to obtain a fraction containing pCE52 DNA. To remove ethidium bromide, the fraction was treated five times with an equal amount of cesium chloride saturated isopropyl alcohol solution consisting of 90% by volume isopropyl alcohol and 10% TES buffer solution. Then, the residue was dialysed against TES buffer solution.

Tryptophan-producing Corynebacterium glutamicum LAR-1 (FERM P-6908, FERM BP-451) was transformed with pTrp 4 - 3 as mentioned above. The thus obtained transformant has been deposited with the American Type Culture Collection as Corynebacterium glutamicum K31, ATCC 39280.

Corynebacterium glutamicum K20, ATCC 39035 containing pTrp 2 - 3 and K31, ATCC 39280 containing pTrp 4 - 3 were tested for production of L-tryptophan as follows:

These strains were cultured with shaking at 30°C in NB medium for 16 hours and 0.5 mℓ of the cultured broth was inoculated in 5 mℓ of the production medium P4 (pH 7.2) consisting of 100 g/ℓ molasses, 20 g/ℓ $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, 0.25 g/ℓ $MgSO_4 \cdot 7H_2O$ and 20 g/ℓ $CaCO_3$ in a test tube. Culturing was carried out with shaking at 30°C for 96 hours.

After culturing, the culture filtrate was subjected to paper chromatography. After ninhydrin color reaction, the amount

of L-tryptophan produced was determined by a colorimetric method.

As control strains, LA-105 and LAR-1 were subjected to similar treatment. The results are shown in Table 4.

Table 4

Strain	Amount of L-tryptophan (mg/mℓ)
LA-105	-
LA-105/pTrp2-3 (K20, ATCC 39035)	0.34
LAR-1	0.48
LAR-1/pTrp4-3 (K31, ATCC 39280)	1.12

Example 4

Cloning of the anthranilic acid synthetase gene of Corynebacterium glutamicum ATCC 13032 and production of tryptophan in Corynebacterium glutamicum:

1) Preparation of the chromosomal DNA:

The chromosomal DNA of Corynebacterium glutamicum ATCC 13032 was prepared by the following method.

The chromosomal DNA was prepared by the same method as in Example 3(1) except that Corynebacterium glutamicum ATCC 13032 was used in place of Brevibacterium flavum ATCC 14067.

2) Cloning of the anthranilic acid synthetase gene:

A ligation mixture was obtained by the same method as in Example 3(2) using 3 μg of pCE52 plasmid DNA prepared in Example 3(4) and 9 μg of the chromosomal DNA prepared above.

3) Transformation of the recombinant plasmid:

Transformants were obtained by the same method as in Example 3(3) using the ligation mixture prepared above.

Plasmid DNAs were isolated from the cultured cells of these transformants in the same way as mentioned above. The

plasmid pTrp 9 - 1, recovered from one of the transformants, was analyzed by digestion with various restriction endonucleases and agarose gel electrophoresis. As a result, the plasmid pTrp 9 - 1 was found to contain a SalI DNA fragment of about 7.3 Kb inserted into the unique SalI site of pCE52.

Strain LA 105 was retransformed with pTrp 9 - 1 in the same way as mentioned above. The colonies grown on RCGP agar medium containing 100 µg/mℓ tryptophan and 400 µg/mℓ kanamycin did not require anthranilic acid for growth and they had the same plasmid as pTrp 9 - 1 characterized by the cleavage pattern by SalI.

The result shows that the gene coding for anthranilic acid synthetase of Corynebacterium glutamicum ATCC 13032 is present in the cloned SalI DNA fragment of about 7.3 Kb and expressed in Corynebacterium glutamicum LA 105.

4) Recovering of plasmid pTrp 13 - 2 resistant to a tryptophan analog from a strain containing pTrp 9 - 1:

LA-105 strain containing pTrp 9 - 1 was grown in NB medium containing 10 µg/mℓ kanamycin to a late stage of the logarithmic growth phase. Cells were recovered by centrifugation and washed with 50 mM Tris-malate buffer (pH 6.0) two times. The washed cells were incubated at room temperature in 50 mM Tris-malate buffer (pH 6.0) containing 400 µg/mℓ N-methyl-N'-nitro-N-nitrosoguanidine for 30 minutes. The treated cells were washed with 50 mM Tris-malate buffer (pH 6.0) and centrifuged two times. The washed cells were cultured at 30°C in NB medium containing 10 µg/mℓ kanamycin for 16 hours. Plasmid DNAs were isolated by the same method as in Example 3.

LA-105 strain was transformed using the isolated plasmid DNAs by the same method as in Example 3. Selection of transformants was carried out on RCGP agar medium containing 0.5 mg/mℓ 4-methyltryptophan or 0.5 mg/mℓ 6-fluorotryptophan, with or without 200 µg/mℓ kanamycin.

Colonies grown on M1 agar medium containing 0.5 mg/mℓ of the corresponding tryptophan analog and on NB agar medium containing 10 µg/mℓ kanamycin were selected.

The thus obtained transformants have the plasmid harboring a gene coding for the anthranilic acid synthetase insensitive to tryptophan. The anthranilic acid synthetase encoded by one of the plasmids, pTrp 13 - 2 was inhibited 50% with 0.25 mM tryptophan, which was 40-fold higher than the concentration of tryptophan (0.006 mM) enough to inhibit 50% the anthranilic acid synthetase encoded by pTrp 9 - 1.

Corynebacterium glutamicum LAR-1 was transformed with pTrp 13 - 2 by the same method as in Example 3. The thus obtained transformant has been deposited with the American Type Culture Collection as Corynebacterium glutamicum K37, ATCC 39285.

5) Production of tryptophan by the transformant:

Corynebacterium glutamicum K37, ATCC 39285 containing pTrp 13 - 2 was tested for production of tryptophan as follows:

The strain was cultured with shaking at 30°C in NB medium for 16 hours and 0.5 mℓ of the cultured broth was inoculated in 5 mℓ of the production medium P4. Culturing was carried out with shaking at 30°C for 96 hours.

After culturing, the culture filtrate was subjected to paper chromatography. After ninhydrin color reaction, the amount of L-tryptophan produced was determined by a colorimetric method.

LAR-1 strain and LAR-1/pTrp 9 - 1 strain were used as control strains. LAR-1/pTrp 9 - 1 was prepared by the same transformation of LAR-1 strain with pTrp 9 - 1 as mentioned above. The results are shown in Table 5.

Table 5

Strain	Amount of L-tryptophan (mg/mℓ)
LAR-1	0.4
LAR-1/pTrp 9 - 1	0.7
LAR-1/pTrp 13 - 2	1.2

Example 5

Cloning of a gene coding for chorismate mutase and prephenate dehydratase derived from Corynebacterium glutamicum K38 strain and production of phenylalanine by the expression of the gene in Corynebacterium glutamicum:

1) Preparation of the chromosomal DNA and plasmid pCG11:

The chromosomal DNA of Corynebacterium glutamicum K38 FERM P-7087 was prepared by the following method:

A seed culture was inoculated into 400 mℓ of SSM. Culturing was carried out with shaking at 30°C. NB medium was used as seed culture. The optical density (OD) at 660 nm was monitored with a Tokyo Koden colorimeter and penicillin G was added at an OD value of 0.2 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of about 0.6.

Cells were harvested from the culture broth and washed with TES buffer. The cells were suspended in a lysozyme solution (pH 8.0) consisting of 25% sucrose, 0.1M NaCl, 0.05M Tris and 0.8 mg/mℓ lysozyme (the same lysozyme solution was used hereinafter) to make 10 mℓ of a suspension which was allowed to react at 37°C for 4 hours. High molecular chromosomal DNAs were isolated from the cells by the method of Saito et al.

Separately pCG11 used as a vector was prepared by the method described in Example 1.

2) Cloning of the gene responsible for the phenotype resistant to para-fluorophenylalanine (PFP):

In this step, 5 units of restriction enzyme BglII was added to 100 μℓ of a BglII reaction solution containing 3 μg of pCG11 plasmid DNA, and 5 units of BamHI was added to 100 μℓ of restriction enzyme BamHI reaction solution (pH 8.0) consisting of 10 mM Tris, 7 mM $MgCl_2$, 100 mM NaCl, 2 mM mercaptoethanol and 0.01% bovine serum albumin (the same BamHI reaction solution was used hereinafter) and containing 9 μg of the chromosomal DNA. The mixtures were allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reactions. The reaction mixtures were mixed with each other. 40 μℓ of a T4

ligase buffer solution II, 40 μℓ of 5 mM ATP, 0.4 μℓ of T4 ligase and 120 μℓ of H_2O were added. The mixture was allowed to react at 12°C for 16 hours.

3) Transformation with recombinant plasmids:

The above ligation mixture was provided for the following transformation. As the recipient for the transformation, Corynebacterium glutamicum L-15 ATCC 31834 was used. The seed culture of L-15 strain was inoculated into NB medium and culturing was carried out with shaking at 30°C. Cells were harvested at an OD value of 0.6. The cells were suspended at about 10^9 cells/mℓ in RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and stirred slowly at 30°C for 5 hours to obtain protoplasts.

Then, 0.5 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 5 minutes. The protoplasts were resuspended in 1 mℓ of TSMC buffer and again subjected to centrifugation and washing. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. 100 μℓ of a mixture (1 : 1 by volume) of a two-fold concentrated TSMC buffer and the ligated DNA mixture described above was added to the protoplast suspension. Then, 0.8 mℓ of a solution containing 20% PEG 6,000 in TSMC buffer solution was added to the mixture. After 3 minutes, 2 mℓ of RCGP medium (pH 7.2) was added and the mixture was centrifuged at 2,500 x g for 5 minutes. The supernatant fluid was removed and the protoplasts were suspended in 1 mℓ of RCGP medium. Then, 0.2 mℓ of the suspension was spread on RCGP agar medium containing 200 μg/mℓ spectinomycin and incubated at 30°C for 7 days.

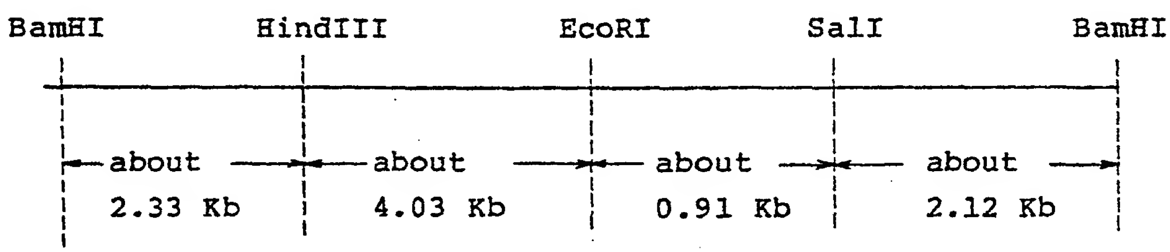
All the spectinomycin-resistant colonies formed on the selection plate were scraped, washed with physiological saline solution and centrifuged two times. The cells were spread on a minimal agar medium M1 containing 100 μg/mℓ spectinomycin and 0.5 mg/mℓ PFP and incubated at 30°C for 2 days. The transformants which are resistant to spectinomycin and PFP were obtained from the colonies formed.

Plasmid DNAs were isolated from cells of these transformants. The plasmid pCS-CM1, recovered from one of the transformants was analyzed by digestion with various restriction endonucleases and analyzed by agarose gel electrophoresis. The analysis showed that a BamHI DNA fragment of about 9.4 Kb was inserted into the unique BglII cleavage site of pCG11 in pCS-CM1.

Furthermore, the same ligation mixture was used to transform a <u>Corynebacterium glutamicum</u> strain requiring phenylalanine and tyrosine for its growth (defective in chorismate mutase and prephenate dehydratase) by the same method described above. Transformants resistant to spectinomycin and non-requiring phenylalanine and tyrosine were selected.

Plasmid DNAs were isolated from these transformants as mentioned above. A plasmid pCS-CM2 obtained from one of the transformants was digested with restriction endonucleases and analysed by agarose gel electrophoresis. The analysis showed that a BamHI DNA fragment of about 9.4 Kb was inserted into the unique BglII cleavage site of pCG11 in pCS-CM2.

Further, digestions with various restriction endonucleases such as EcoRI, SalI, HindIII and the like gave the same cleavage patterns in pCS-CM1 and pCS-CM2, indicating that both plasmids have the same physical structure as shown below.



When a phenylalanine- and tyrosine-requiring <u>Corynebacterium glutamicum</u> strain was transformed with pCS-CM1 and pCS-CM2 by the same method mentioned above. The resulting transformants grown on RCGP agar medium containing 100 μg/mℓ each phenylalanine and tyrosine and 200 μg/mℓ spectinomycin all exhibited phenylalanine- and tyrosine-prototrophy and resistance

to PFP, and carried the same plasmids as pCS-CM1 and pCS-CM2.

These results indicate that the BamHI DNA fragment of about 9.4 Kb cloned in pCS-CM1 and pCS-CM2 contains the genes encoding for chorysmate mutase and prephenate dehydratase of Corynebacterium glutamicum K38 and that the DNA fragment can confer resistance to para-fluorophenylalanine on Corynebacterium glutamicum.

4) Production of phenylalanine by the transformant:

Corynebacterium glutamicum K38 (FERM P-7087, FERM BP-454) which produces phenylalanine was transformed with pCS-CM2 as mentioned above. The thus obtained transformant has been deposited with the Fermentation Research Institute as Corynebacterium glutamicum K39, FERM P-7088 (FERM BP-459).

Corynebacterium glutamicum K39, carrying pCS-CM2 was tested for L-phenylalanine production as follows.

The strain was cultured in NB medium at 30°C for 16 hours and 0.5 mℓ of the culture liquor was inoculated in 5 mℓ of a production medium P4. Culturing was carried out at 30°C for 96 hours. The culture filtrate was subjected to paper chromatography and color reaction with ninhydrin. The amount of L-phenylalanine formed was determined colorimetrically. As a control, Corynebacterium glutamicum K38 was treated as mentioned above. The results are shown in Table 6.

Table 6

Strain	Amount of L-phenylalanine (mg/mℓ)
Corynebacterium glutamicum K38	6.0
Corynebacterium glutamicum K39	9.6

Example 6

(1) Cloning of a DNA fragment containing Escherichia coli threonine operon:

Cloning was carried out using a host-vector system of

Escherichia coli. Plasmid pGA22, used as a vector, was isolated from cultured cells of a derivative of Escherichia coli K12 carrying the present plasmid according to the method of An [An, G. et al.: J. Bacteriol., 140, 400 (1979)]. The chromosomal DNA used as a donor DNA was isolated from cultured cells of Escherichia coli K12 Hfr (ATCC 23740) by the phenol-extraction method of Smith [Smith, M.G.: Methods in Enzymology, 12, part A, 545 (1967)].

Then, 0.4 unit of HindIII (16 units/μℓ) was added to 60 μℓ of a HindIII reaction solution (pH 7.5) consisting of 10 mM Tris, 7 mM $MgCl_2$ and 60 mM NaCl (the same HindIII reaction solution was used hereinafter) and containing 4 μg of pGA22 plasmid DNA. The mixture was allowed to react at 37°C for 30 minutes and heated at 65°C for 10 minutes to stop the reaction. pGA22, which has two HindIII cleavage sites, was digested with HindIII under the same conditions and subjected to agarose gel electrophoresis. It was confirmed that one of the two HindIII cleavage sites present in pGA22 was cleaved. Separately, 4 units of HindIII was added to 140 μℓ of the HindIII reaction solution containing 8 μg of the chromosomal DNA. The mixture was allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction. Then, 40 μℓ of the T4 ligase buffer solution II, 40 μℓ of 5 mM ATP, 0.3 μℓ of T4 ligase and 120 μℓ of H_2O were added to a mixture of the above digests and reaction was carried out at 12°C for 16 hours. The reaction mixture was extracted twice with 400 μℓ of phenol saturated with TES buffer solution and subjected to dialysis against TES buffer solution to remove phenol.

The ligase reaction mixture was used to transform Escherichia coli K-12, GT-3 (J. Bacteriol. 117, 133, 1974) which is a mutant defective in three aspartate kinases and requiring homoserine and diaminopimelic acid. Competent cells of the GT-3 strain were prepared according to the method of Dagert, M. et al., Gene, 6, 23 (1979). That is, the strain was inoculated in 50 mℓ of L-medium (pH 7.2) consisting of 10 g/ℓ Bacto-trypton, 5 g/ℓ yeast extract, 1 g/ℓ glucose and 5 g/ℓ sodium chloride and containing 100 μg/mℓ diaminopimelic acid and cultured at 37°C to

an optical density (OD) value at 660 nm of 0.5. The culture was cooled with ice water for 10 minutes and cells were recovered by centrifugation. The cells were suspended in 20 mℓ of cooled 0.1M calcium chloride. The suspension was allowed to stand at 0°C for 20 minutes and then centrifuged to recover the cells. The cells were suspended in 0.5 mℓ of 0.1M calcium chloride and allowed to stand at 0°C for 18 hours. Then, 200 μℓ of the ligase reaction mixture mentioned above was added to 400 μℓ of the cell suspension treated with calcium chloride. The mixture was allowed to stand at 0°C for 10 minutes and then heated at 37°C for 5 minutes. Thereafter, 9 mℓ of the L-medium was added and the mixture was incubated with shaking at 37°C for 2 hours.

Cells were recovered by centrifugation and washed with a physiological saline solution twice. The cells were spread on M9 minimal agar medium (pH 7.2) consisting of 2 g/ℓ glucose, 1 g/ℓ NH_4Cl, 6 g/ℓ Na_2HPO_4, 3 g/ℓ KH_2PO_4, 0.1 g/ℓ $MgSO_4 \cdot 7H_2O$, 15 mg/ℓ $CaCl_2 \cdot 2H_2O$, 4 mg/ℓ thiamine hydrochloride and 15 g/ℓ agar and containing 12.5 μg/mℓ kanamycin (the same M9 minimal agar medium was used hereinafter). Culturing was carried out at 37°C for 3 days. Only one colony was formed and the cells from this colony could also grow on an agar medium containing 25 μg/mℓ ampicillin, 25 μg/mℓ chloramphenicol or 25 μg/mℓ kanamycin, which is a selection marker of pGA22.

A plasmid DNA was isolated from cultured cells of the transformant by the same method as in the isolation of pGA22. The plasmid DNA was digested with restriction endonucleases and analyzed by agarose gel electrophoresis. The plasmid DNA had the structure illustrated as pGH2 in Fig. 1. Since the DNA fragment inserted in pGA22 had the same cleavage sites for restriction endonucleases as the cloned DNA fragment containing *Escherichia coli* operon (Cossart, P. *et al*.: Molec. Gen. Genet., 175, 39, 1979), it is confirmed that pGH2 had the threonine operon.

(2) *In vitro* recombination of pCG11 and pGH2

2 units of BglII (6 units/μℓ) was added to 100 μℓ of the BglII reaction buffer solution containing 2 μg of pCG11

plasmid DNA obtained by the same method as in Example 1. The mixture was allowed to react at 37°C for 60 minutes. Separately, 2 units of BamHI (6 units/μℓ) was added to 100 μℓ of BamHI reaction buffer polution containing 2 μg of pGH2 plasmid DNA. The mixture was allowed to react at 37°C for 60 minutes. Both digests were heated at 65°C for 10 minutes, and mixed. Then, 40 μℓ of T4 ligase buffer solution II, 40 μℓ of 5 mM ATP, 0.2 μℓ of T4 ligase and 120 μℓ of H_2O were added to the whole mixture of both the digests. Reaction was carried out at 12°C for 16 hours. The reaction mixture was extracted twice with 400 μℓ of phenol saturated with TES buffer solution and subjected to dialysis against TES buffer solution to remove phenol.

(3) Recovery of plasmid pEthr1

Protoplasts of Corynebacterium glutamicum LA201 which requires homoserine and leucine were used for transformation. A seed culture of Corynebacterium glutamicum LA201 was inoculated in NB medium and cultured with shaking at 30°C. Cultured cells were collected at an OD value of 0.6 and suspended in an RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme at a concentration of about 10^9 cells/mℓ. The suspension was put into an L-tube and allowed to react with gentle shaking at 30°C for 5 hours to make protoplasts.

Then, 0.5 mℓ of the protoplast suspension was put into a small tube and centrifuged at 2,500 x g for 5 minutes to obtain pellets. The pellets was resuspended in 1 mℓ of a TSMC buffer solution, and subjected to centrifugation and washing. The protoplasts were resuspended in 0.1 mℓ of the TSMC buffer solution. Then, 100 μℓ of a mixture of a two-fold concentrated TSMC buffer solution and the above-described DNA mixture treated with ligase (1:1) was added to the suspension and 0.8 mℓ of a TSMC buffer solution containing 20% PEG 6,000 was added. After 3 minutes, 2 mℓ of the RCGP medium (pH 7.2) was added and the mixture was centrifuged at 2,500 x g for 5 minutes. The supernatant fluid was removed and the precipitated protoplasts were suspended in 1 mℓ of the RCGP medium. The suspension was slowly shaken at 30°C for 2 hours. Then, 0.1 mℓ of the

susp nsion was spread on RCGP agar medium containing 400 μg/mℓ kanamycin, and culturing was carried out at 30°C for 6 days.

Kanamycin-resistant transformants grown over the whole surface of the agar medium were scraped, washed with physiological saline solution and subjected to centrifugation. The cells were spread on a minimal agar medium M1 containing 50 μg/mℓ leucine. Culturing was carried out at 30°C for 3 days. Among colonies developed, those able to grow on the NB agar medium containing 20 μg/mℓ kanamycin and 100 μg/mℓ spectinomycin were obtained.

Three strains selected at random were grown in 400 mℓ of NB medium to an OD value of about 0.8. Cells were harvested and the plasmids were isolated from the cells by ethidium bromide-cesium chloride density gradient centrifugation described in Example 1 whereby 40 to 55 μg of plasmid DNA were recovered from each strain.

These plasmid DNAs were digested with restriction endonucleases and analyzed by agarose gel electrophoresis to determine the molecular weights and cleavage sites for PstI, EcoRI and XhoI. The plasmid obtained from one strain is named pEthr1 and the structure is illustrated in Fig. 2. It was confirmed that pEthr1 has the structure wherein a BamHI fragment containing pGH2 threonine operon is inserted into pCG11 at its BglII site. One of the remaining strains has the same plasmid as pEthr1 and the other has a plasmid wherein the BamHI fragment containing pGH2 threonine operon is combined at the opposite orientation.

<u>Corynebacterium</u> <u>glutamicum</u> LA201 strain was again transformed with these plasmid DNAs as mentioned above. As a result, strains which do not require homoserine were obtained at high frequency, about 10^{-3} cell/regenerated cell. All of them are endowed with the phenotypes of the resistance to kanamycin and spectinomycin and have the same plasmid as the donor plasmid characterized by the cleavage pattern for various restriction endonucleases.

(4) Production of L-isoleucine by the pEthr1-carrying strain:

The protoplasts of Corynebacterium glutamicum K40 and Brevibacterium flavum ATCC 14067 were transformed with pEthr1. Corynebacterium glutamicum K40 (FERM P-7160, FERM BP-455) and Brevibacterium flavum ATCC 14067 were cultured with shaking in NB medium at 30°C for 16 hours, and 0.1 mℓ of the seed culture was inoculated into 10 mℓ of SSM medium in an L-tube. Culturing was carried out at 30°C in a Monod-type culture bath, and penicillin G was added at an OD value of 0.15 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of about 0.6. Cells were harvested and suspended in 2 mℓ of RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and stirred slowly at 30°C for 14 hours to obtain protoplasts.

Then, 1 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 15 minutes. The protoplasts were resuspended in 1 mℓ of TSMC buffer and centrifuged at 2,500 x g. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. One hundred microliter of a mixture (1:1 by volume) of a two-fold concentrated TSMC buffer and the pEthr1 DNA solution isolated above was added to the protoplast suspension. Transformation was carried out using PEG 6,000 by the same method described in Example 1 for expression of the desired gene. Then, 0.1 mℓ of the mixture was spread on RCGP agar medium containing 400 μg/mℓ spectinomycin and incubated at 30°C for 10 days. From the colonies developed, the transformants which grow on NB agar medium containing 100 μg/mℓ spectinomycin and 20 μg/mℓ kanamycin were obtained. The strains resistant to spectinomycin and kanamycin were cultured with shaking in 400 mℓ of SSM medium, and penicillin G was added at an OD value of 0.15 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of 0.65, and cells were harvested. From the cells, plasmids were isolated by the same method as the isolation method of pCG11 in Example 1. These plasmids were digested with restriction endonucleases and analyzed by agarose gel

electrophoresis. The analysis showed that some of the plasmids have the same physical structure as pEthr1 characterized by the cleavage pattern for various restriction endonucleases. Such transformants are Corynebacterium glutamicum K41 (FERM P-7161, FERM BP-456) and Brevibacterium flavum K42 (FERM BP-355).

Corynebacterium glutamicum K40, Brevibacterium flavum ATCC 14067 and their pEthr1-carrying strains were tested for L-isoleucine production as follows. The strain was cultured with shaking in NB medium at 30°C for 16 hours and 0.5 mℓ of the seed culture was inoculated into a production medium adjusted to pH 7.2 consisting of 100 g/ℓ glucose, 20 g/ℓ $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, 1 g/ℓ $MgSO_4 \cdot 7H_2O$, 10 mg/ℓ $FeSO_4 \cdot 7H_2O$, 10 mg/ℓ $MnSO_4 \cdot (4\text{-}6)H_2O$, 100 μg/ℓ biotin and 30 g/ℓ $CaCO_3$ in a test tube. Culturing was carried out with shaking at 30°C for 72 hours. The culture filtrate was subjected to paper chromatography and color reaction with ninhydrin. The amount of L-isoleucine formed was determined colorimetrically. The results are shown in Table 7.

Table 7

Strain	Amount of L-isoleucine (mg/mℓ)
Corynebacterium glutamicum K40	1.2
Corynebacterium glutamicum K41	2.7
Brevibacterium flavum ATCC 14067	0
Brevibacterium flavum K42	0.8

Example 7

Production of tyrosine:

(1) Preparation of the chromosomal DNA and plasmid DNA:

The chromosomal DNA of Escherichia coli JA194 [Proc. Natl. Acad. Sci., 94, 487-491 (1977)] was prepared by the following method.

A seed culture was inoculated into 400 mℓ of LB (pH 7.0, the same LB was used hereinafter). Culturing was carried

out with shaking at 37°C and continued to a latter stage of the logarithmic growth phase. Cells were harvested from the culture broth and high molecular chromosomal DNAs were isolated from the cells by the method of Saito et al.

Separately pBR322 used as a vector was prepared from the cultured cells of Escherichia coli JA194 harboring pBR322 by the following method.

The strain was grown with shaking at 37°C in 400 mℓ of LB containing 100 μg/mℓ ampicillin to a latter stage of the logarithmic growth phase. Cells were harvested from the culture broth and lysed by the method of Tanaka, et al., [J. Bacteriol. 121, 354-362 (1975)].

The whole lysate was centrifuged at 4°C at 28,000 rpm for one hour. The supernatant fluid was recovered and one fifth volume of 50% (W/V) polyethyleneglycol (PEG) 6,000 aqueous solution was added. The mixture was stirred slowly and allowed to stand at 4°C overnight. Formed precipitates were collected by centrifugation at 3,000 rpm at 4°C for 5 minutes and dissolved in 5 mℓ of TE buffer solution (pH 7.5) consisting of 10 mM Tris and 1 mM EDTA·Na_2 (the same TE buffer solution was used hereinafter). Then, one mℓ of 1.5 mg/mℓ ethidium bromide was added, and TE buffer solution was added to the total volume of 7.5 mℓ. To the mixture was added 7.875 g of CsCl, and dissolved completely. The solution was centrifuged at 105,000 x g at 20°C for 40 hours. A plasmid band detected under UV irradiation was taken out with an injector. Ethidium bromide was extracted three times with isopropanol containing 15% (V/V) TE buffer solution. The residue was dialysed against TE buffer solution at 4°C overnight, and used as a plasmid DNA.

(2) Cloning of a DNA fragment containing the gene coding for DAHPase, CMase and PDGase:

In this step, 5 units each of restriction enzymes EcoRI and HindIII were added to 100 μℓ of a HindIII reaction solution containing 3 μg of pBR322 plasmid DNA prepared above, and 5 units each of EcoRI and HindIII was added to 100 μℓ of restriction enzyme HindIII reaction solution containing 9 μg of

the chromosomal DNA. Each of the mixtures was allowed to react at 37°C for 60 minutes and heated at 65°C for 10 minutes to stop the reaction. Both of the mixtures were admixed with each other. Then, 40 μℓ of a T4 ligase buffer solution II, 40 μℓ of 5 mM ATP, 0.4 μℓ of T4 ligase and 120 μℓ of H_2O were added. The mixture was allowed to react at 12°C for 16 hours.

The above ligation mixture was provided for the following transformation. As the recipient for the transformation, DAHPase-deficient Escherichia coli AB3248 strain [J. Bact., 93, 237-244 (1967)] or tyrA gene (CMase)-deficient Escherichia coli AT2273 strain [J. Bact., 91, 1494 (1966)] was used. The seed culture of the strain was inoculated into LB and competent cells were prepared according to the method of M. Dagert, [Gene, 6, 23-28 (1979)].

Fifty microliter of the ligation mixture was added to 0.2 mℓ of a solution containing 10^9/mℓ competent cells and the mixture was allowed to stand under ice cooling for 10 minutes. After heating at 37°C for 5 minutes, 2 mℓ of LB was added, followed by standing at 37°C for 90 to 120 minutes. Cells were subjected to washing with physiological saline solution and centrifugation twice. The cells were spread on M9 plate medium (pH 7.0) consisting of 1 g/ℓ NH_4Cl, 6 g/ℓ Na_2HPO_4, 3 g/ℓ KH_2PO_4, 5 g/ℓ NaCl, 0.1 g/ℓ $MgSO_4 \cdot 7H_2O$, 0.015 g/ℓ $CaCl_2 \cdot 2H_2O$, 3 g/ℓ glucose, 4 mg/ℓ vitamine B1 and 15 g/ℓ agar, [J. Bact. 121, 354-362 (1975), the same M9 plate medium was used hereinafter] and containing 50 μg/mℓ each histidine, proline, arginine, isoleucine and valine. Colonies grown on M9 plate medium were plated on LB plate media containing 100 μg/mℓ ampicillin and 20 μg/mℓ tetracycline, respectively. Colonies which grew on the ampicillin-containing medium and did not grow on the tetracycline-containing medium were selected. Plasmid DNAs were isolated from the thus selected transformants which grew on M9 plate medium containing histidine, proline, arginine, isoleucine and valine and which were resistant to ampicillin and sensitive to tetracycline by the same method as described above. Plasmid pEaroF1 isolated from one of the transformants was digested with various restriction endonucleases and analysed by agarose gel

electrophoresis. The analysis showed that an EcoRI-HindIII cleaved DNA fragment of about 4.2 Kb was inserted into the larger EcoRI-HindIII cleaved fragment of pBR322.

Escherichia coli. AB3248 and Escherichia coli AT2273 were transformed by the same method as described above using pEaroFl obtained above. Both transformants grew on M9 medium containing histidine, proline, arginine, isoleucine and valine and simultaneously were resistant to ampicillin. The fact showed that the transformants had the same plasmids as pEaroFl.

The results described above showed that the genes encoding for DAHPase, CMase and PDGase exist on the DNA fragment of about 4.2 Kb cloned in pEaroFl.

Further, pEaroFl having cleavage sites with various restriction endonucleases such as EcoRI, BamHI, HindIII and the like as shown in Fig. 3 was compared with plasmid pKB45 reported by G. Zurawski, Proc. Natl. Acad. Sci. USA 75, 4271 (1978) to show that the DNA fragment of about 4.2 Kb in pEaroFl possesses aroF, tyrA and pheA genes of Escherichia coli.

(3) Subcloning of aroFtyrA gene:

A DNA fragment containing aroFtyrA gene was recovered from plasmid pEaroFl DNA prepared above and ligated to pCE51 which is a shuttle vector for Escherichia coli and Corynebacterium glutamicum.

pCE51 is a recombinant plasmid wherein plasmid pCGl (Japanese Published Unexamined Patent Application No. 134500/82) of Corynebacterium glutamicum is ligated with plasmid pGA22 of Escherichia coli [refer to An, G. et al: J. Bacteriol., 140, 400 (1979)]. The ligation is carried out using the same cohesive ends of BglII cleaved pCGl and BamHI fragment of pGA22 containing the kanamycin-resistant gene.

pCE51 is practically prepared by the method described in Japanese Published Unexamined Patent Application Nos. 105999/83 and 126789/83.

Five units of HincII was added to 100 μl of HincII reaction solution consisting of 10 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$ and 60 mM NaCl and containing 3 μg of plasmid pEaroFl DNA

and reaction was carried out at 37°C for 60 minutes. Three-tenth units of HincII was added to 100 μℓ of HincII reaction solution containing 3 μg of plasmid pCE51 DNA prepared from pCE51-carrying Escherichia coli JA194 strain by the same method as mentioned above and reaction was carried out at 37°C for 60 minutes to cut pCE51 at one of two HincII cleavage sites. Both of the reaction mixtures were mixed with each other. 40 μℓ of T4 ligase buffer solution II, 40 μℓ of 5 mM ATP, 0.4 μℓ of T4 ligase and 120 μℓ of water were added. The mixture was allowed to react at 12°C for 16 hours and reaction was stopped by heating at 65°C for 10 minutes.

The ligation mixture was provided for the following transformation. As the recipient for the transformation, Escherichia coli AB3248 strain was used. Transformation was carried out by the same method as described above to obtain colonies grown on M9 plate medium containing histidine, proline, arginine, isoleucine and valine. Colonies grown on LB plate medium containing 20 μg/mℓ kanamycin were selected from the colonies obtained above.

Plasmid DNAs were isolated from the transformants which grew on M9 plate medium containing histidine, proline, arginine, isoleucine and valine and were resistant to kanamycin by the same method as described above. Plasmid pKmlaroF1 obtained from one of the transformants were digested with various restriction endonucleases and analysed by agarose gel electrophoresis. The analysis showed that a HincII cleaved DNA fragment of about 3.8 Kb bearing aroFtyrA of pEaroF1 was inserted in one of two HincII cleavage sites of pCE51.

Plasmid pKmlaroF1 obtained above has the restriction pattern as illustrated in Fig. 4.

(4) Preparation of tyrosine- and tyrosine analog-resistant plasmid pKmlaroF1-m-18 from pKmlaroF1-carrying strain:

pKmlaroF1-carrying AB3248 strain was grown in LB medium containing 20 μg/mℓ kanamycin to a latter stage of the logarithmic growth phase. Cells were harvested by centrifugation and washed with 50 mM Tris-malate buffer solution

(pH 6.0) twice and incubated with 400 μg/mℓ N-metyl-N'-nitro-N-nitrosoguanidine in 50 mM Tris-malate buffer solution (pH 6.0) at room temperature for 30 minutes. The treated cells were harvested by centrifugation and washed with 50 mM Tris-malate buffer solution (pH 6.0) twice. The washed cells were cultured in LB medium containing 20 μg/mℓ kanamycin at 30°C for 16 hours and plasmid DNAs were isolated by the same method as mentioned above.

Escherichia coli AB3248 was transformed using the isolated plasmids. Selection of transformants was carried out on M9 plate medium containing 0.25 mg/mℓ tyrosine and 50 μg/mℓ each histidine, proline, arginine, isoleucine and valine. Colonies grown on M9 plate medium containing 0.25 mg/mℓ tyrosine and 50 μg/mℓ each histidine, proline, arginine, isoleucine and valine and on LB plate medium containing 20 μg/mℓ kanamycin were selected from the developed colonies.

The thus obtained colonies were resistant to 3-aminotyrosine (3AT), a tyrosine analog (product of Shigma Co.) since they could grow on M9 plate medium containing 0.2 mg/mℓ 3AT and 50 μg/mℓ each histidine, proline, arginine, isoleucine and valine.

The plasmids obtained from the thus prepared transformant can confer resistance to tyrosine or a tyrosine analog on a microorganism. Escherichia coli AB3248 strain carrying one of such plasmids, pKmlaroF-m-18 was able to grow on M9 plate medium containing 1 mg/mℓ tyrosine or 0.5 mg/mℓ 3AT, and 50 μg/mℓ each histidire, proline, arginine, isoleucine and valine.

(5) Transformation of Corynebacterium glutamicum K43 with pKmlaroF1 and pKmlaroF1-m-18:

A seed culture of Corynebacterium glutamicum K43 (FERM P-7162, FERM BP-457) was inoculated in Semi-synthetic medium SSM containing 50 μg/mℓ phenylalanine and culturing was carried out with shaking at 30°C. NB medium was used for seed culture. The optical density (OD) at 660 nm was monitored with a Tokyo Koden colorimeter and, at an OD value of 0.2, penicillin G was added

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to the broth to a final concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of about 0.6.

Cells were harvested at an OD value of 0.6. The cells were suspended at about 10^9 cells/mℓ in RCGP medium containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and stirred slowly at 30°C for 5 hours to obtain protoplasts. Then, 0.5 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 5 minutes. The protoplasts were resuspended in 1 mℓ of TSMC buffer and again subjected to centrifugation and washing. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. One hundred microliter of a mixture (1 : 1 by volume) of a two-fold concentrated TSMC buffer and the ligated DNA mixture described above was added to the protoplast suspension. Then, 0.8 mℓ of a solution containing 20% PEG 6,000 in TSMC buffer solution was added to the mixture. Plasmid DNAs, pKmlaroFl and pKmlaroFl-m-18 were prepared from Escherichia coli AB3248 carrying these plasmids as described above. After 3 minutes, 2 mℓ of RCGP medium (pH 7.2) was added and the mixture was centrifuged at 2,500 x g for 5 minutes. The supernatant fluid was removed and the precipitated protoplasts were suspended in 1 mℓ of RCGP medium. Then, 0.2 mℓ of the suspension was spread on RCGP agar medium containing 200 μg/mℓ kanamycin and incubated at 30°C for 7 days. Colonies resistant to kanamycin were grown on the selection plate.

(6) Production of tyrosine by the transformant:

The thus obtained transformants carrying pKmlaroFl and pKmlaroFl-m-18 have been deposited with the Fermentation Research Institute as Corynebacterium glutamicum K44 (FERM P-7163, FERM BP-458) and Corynebacterium glutamicum K45 (FERM P-7164, FERM BP-460), respectively.

L-tyrosine production by pKmlaroFl and pKmlaroFl-m-18-carrying strains was carried out as follows.

The strain was cultured in NB aqueous medium at 30°C for 16 hours and 0.5 mℓ of the culture broth was inoculated in 5 mℓ of a production medium P4 containing 0.25% NZ amine.

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Culturing was carried out at 30°C for 96 hours.

After culturing, 6N NaOH solution was added to the broth to a concentration of 50 μℓ/mℓ and the mixture was heated at 65°C for 5 minutes to dissolve precipitated tyrosine completely. The culture filtrate was subjected to paper chromatography and color reaction with ninhydrin, and the amount of L-tyrosine formed was determined colorimetrically. As a control, Corynebacterium glutamicum K43 was similarly treated. The results are shown in Table 8.

Table 8

Strain	Amount of L-tyrosine (mg/mℓ)
Corynebacterium glutamicum K43	4.8
Corynebacterium glutamicum K44	5.3
Corynebacterium glutamicum K45	7.7

Example 8

(1) In vitro recombination of pLC20-10 and pCE53:

pLC20-10 was isolated from cultured cells of a derivative of Escherichia coli K-12 carrying the present plasmid according to the method of An [An, G. et al.: J. Bacteriol., 140, 400 (1979)]. pCE53 was isolated by the method described in Example 3.

Five units of PstI (5 units/μℓ) and 5 units of BamHI (5 units/μℓ) were added to 30 μℓ of PstI-BamHI reaction buffer solution (pH 8.0) consisting of 15 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 25 mM $(NH_4)_2SO_4$, 1 mM mercaptoethanol and 0.01% bovine serum albumin containing 5 μg of pLC20-10 plasmid DNA. The mixture was allowed to react at 33°C for 90 minutes. Five microgram of pCE53 plasmid DNA was treated by the same method as in the treatment of pLC20-10. Both digests were heated at 65°C for 10 minutes and mixed. Then, 10 μℓ of T4 ligase buffer II, 1 μℓ of 40 mM ATP, 0.3 μℓ of T4 ligase and 30 μℓ of H_2O were added to the whole mixture. The reaction was carried out at 4°C for 12 hours.

(2) Recovery of pEarg1:

Transformation was carried out using Escherichia coli CH754 which is a derivative of Escherichia coli K-12 and requires methionine, tryptophan and arginine (defective mutation of argininosuccinase, argH). Competent cells of CH754 were prepared by the method of Dagert [Dargert, M. et al.: Gene, 6, 23 (1979)]. That is, CH754 strain was inoculated to 50 mℓ of L-broth and culturing was carried out at 37°C to an OD value at 660 nm of 0.5 by Tokyo Koden Colorimeter. The culture broth was cooled on an ice water bath for 10 minutes and centrifuged. Cells were suspended in 20 mℓ of 0.1M $CaCl_2$ cooled and allowed to stand at 0°C for 20 minutes. Cells recovered by centrifugation were suspended in 0.5 mℓ of 0.1M $CaCl_2$ and allowed to stand at 0°C for 18 hours. Fifty microliter of ligation mixture obtained above was added to 150 μℓ of the $CaCl_2$-treated cell suspension. The mixture was allowed to stand at 0°C for 10 minutes and at 37°C for 5 minutes. Then 2 mℓ of L-broth was added and culturing was carried out with shaking at 37°C for 2 hours. Cells were subjected to washing with physiological saline solution and centrifugation twice. The cells were spread on A agar medium containing 40 μg/mℓ methionine, 40 μg/mℓ tryptophan and 25 μg/mℓ kanamycin. Culturing was carried out at 37°C for 3 days. A plasmid DNA was isolated from cultured cells of a developed transformant by the same method as in the isolation of pLC20-10. The plasmid DNA was digested with restriction endonucleases and analyzed by agarose gel electrophoresis. The plasmid, as illustrated in Fig. 5, has a structure wherein PstI-BamHI fragment containing the genes responsible for the biosynthesis of arginine derived from pLC20-10 and PstI-BamHI fragment containing the gene responsible for kanamycin resistance derived from pCE53 were ligated. The plasmid was named pEarg1.

CH754 was retransformed using the plasmid DNA by the same method as described above. Arginine-nonrequiring transformants were obtained at a high frequency and all of them were endowed with the phenotype of kanamycin resistance. In the case that Escherichia coli CSR603 which is defective in

acetylornithine deacetylase (argE) on the pathway of the biosynthesis of arginine and is a derivative of Escherichia coli K-12 was transformed, all of the transformants resistant to kanamycin were endowed with arginine-nonrequiring property.

Corynebacterium glutamicum LA291 requiring arginine for its growth was transformed by pEargl. Corynebacterium glutamicum LA291 is a mutant which is derived by a conventional mutagenesis from lysozyme-sensitive mutant strain L-15 derived from Corynebacterium glutamicum ATCC 31833 and which requires arginine for its growth. It is assumed that the defective mutation depends on the loss of argininosuccinate synthetase corresponding to argG of Escherichia coli or argininosuccinase corresponding to argH of Escherichia coli since growth of the mutant does not respond to citrulline which is a precursor two steps before arginine on the pathway of arginine biosynthesis. A seed culture of Corynebacterium glutamicum LA291 was inoculated in NB medium and culturing was carried out with shaking at 30°C. Cells were harvested at an OD value of 0.6 and suspended in an RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme at a concentration of about 10^9 cells/mℓ. The suspension was put into an L-tube and allowed to react with gentle shaking at 30°C for 5 hours to make protoplasts.

Then, 0.5 mℓ of the protoplast suspension was transferred into a small tube and centrifuged at 2,500 x g for 5 minutes. The pellet was resuspended in 1 mℓ of TSMC buffer solution and centrifuged. The protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. Then, 100 μℓ of a mixture of a two-fold concentrated TSMC buffer solution and the pEargl plasmid DNA solution (1:1) was added to the suspension and 1.0 mℓ of TSMC buffer solution containing 20% PEG 6,000 was added. After 3 minutes, the mixture was centrifuged at 2,500 x g for 5 minutes, and the supernatant fluid was removed. The precipitated protoplasts were suspended in 1 mℓ of RCGP medium (pH 7.4), and the suspension was slowly shaken at 30°C for 2 hours. Then, 0.3 mℓ of the protoplast suspension was spread on RCGP agar medium (pH 7.4), i.e. the RCGP medium supplemented by 1.6% agar, containing 400 μg/mℓ kanamycin, and

culturing was carried out at 30 C for 6 days.

All of the developed kanamycin-resistant transformants were endowed with arginine-nonrequiring property.

A plasmid DNA was isolated from cultured cells of the transformant by the same method as in the isolation of pCE53. The plasmid was digested with restriction endonucleases and analyzed by agarose gel electrophoresis to determine that the plasmid has the same structure as pEargl characterized by the cleavage pattern for various restriction endonucleases.

(3) Production of L-arginine by the pEargl-carrying strains:

The protoplasts of Corynebacterium glutamicum ATCC 13032, Corynebacterium herculis ATCC 13868 and Brevibacterium flavum ATCC 14067 were transformed with pEargl. The strains were cultured with shaking in NB medium at 30°C for 16 hours, and 0.1 mℓ of the seed culture was inoculated into 10 mℓ of SSM medium in an L-tube. Culturing was carried out at 30°C in a Monod-type culture bath, and penicillin G was added at an OD value of 0.15 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of about 0.6. Cells were harvested and suspended in 2 mℓ of RCGP medium (pH 7.6) containing 1 mg/mℓ lysozyme. The suspension was put in an L-tube and stirred slowly at 30°C for 14 hours to obtain protoplasts. Then, 1 mℓ of the protoplast suspension was put in a small test tube and centrifuged at 2,500 x g for 15 minutes. The protoplasts were resuspended in 1 mℓ of TSMC buffer and again subjected to centrifugation at 2,500 x g and washing. The washed protoplasts were resuspended in 0.1 mℓ of TSMC buffer solution. One hundred microliter of a mixture (1:1) of a two-fold concentrated TSMC buffer and the pEargl DNA mixture described above was added to the protoplast suspension. Transformation was carried out using PEG 6,000 by the same method described in Example 1 for expression of the desired gene. Then, 0.3 mℓ of the mixture was spread on RCGP agar medium containing 400 μg/mℓ kanamycin and incubated at 30°C for 10 days.

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Kanamycin-resistant strains were cultured with shaking in 400 mℓ of SSM medium, and penicillin G was added at an OD value of 0.15 to a concentration of 0.5 unit/mℓ. Culturing was continued to an OD value of 0.65, and cells were harvested. From the cells, plasmids were isolated by the same method as the isolation method of pCE53 in Example 3. These plasmids were digested with restriction endonucleases and analyzed by agarose gel electrophoresis. The analysis showed that the plasmids have the same structure as pEargl characterized by the cleavage pattern for various restriction endonucleases. Such transformants are Corynebacterium glutamicum K46 (FERM BP-356), Corynebacterium herculis K47 (FERM BP-367) and Brevibacterium flavum K48 (FERM BP-357).

Corynebacterium glutamicum ATCC 13032, Corynebacterium herculis ATCC 13868, Brevibacterium flavum ATCC 14067 and their pEargl-carrying strains were tested for L-arginine production as follows. The strains were cultured with shaking in NB medium at 30°C for 16 hours and 0.5 mℓ of the seed culture was inoculated in a production medium (pH 7.0) consisting of 80 g/ℓ molasses (as glucose), 40 g/ℓ $(NH_4)_2SO_4$, 0.5 g/ℓ KH_2PO_4, 0.5 g/ℓ K_2HPO_4, and 20 g/ℓ $CaCO_3$. Culturing was carried out with shaking at 30°C for 72 hours. The culture filtrate was subjected to paper chromatography and color reaction with ninhydrin. The amount of L-arginine formed was determined colorimetrically. The results are shown in Table 9.

Table 9

Strain	Amount of L-arginine (mg/mℓ)
Corynebacterium glutamicum ATCC 13032	0
Corynebacterium glutamicum K46	1.6
Corynebacterium herculis ATCC 13868	0
Corynebacterium herculis K47	1.8
Brevibacterium flavum ATCC 14067	0
Brevibacterium flavum K48	1.0

Claims

1. A process for producing an amino acid which comprises transforming a host microorganism belonging to the genus Corynebacterium or Brevibacterium with a recombinant DNA of a DNA fragment containing a gene encoding for the enzyme involved in the biosynthesis of the amino acid and a vector DNA, culturing the transformant in a nutrient medium, accumulating the amino acid in the culture medium and recovering the amino acid therefrom.

2. The process according to claim 1, wherein the amino acid is histidine, tryptophan, phenylalanine, isoleucine, tyrosine or arginine.

3. The process according to claim 1, wherein the DNA fragment containing said gene is derived from prokaryotes, eukaryotes, bacteriophages, viruses or plasmids.

4. The process according to claim 3, wherein the prokaryote is selected from bacteria belonging to the genus Escherichia, Corynebacterium, Brevibacterium, Microbacterium, Bacillus, Staphylococcus, Streptococcus or Serratia.

5. The process according to claim 4, wherein the bacterium is resistant to histidine analog, tryptophan analog, phenylalanine or phenylalanine analog.

6. The process according to claim 5, wherein the bacterium is resistant to 1,2,4-triazole-3-alanine, 4-methyltryptophan, 5-methyltryptophan and 6-methyltryptophan or para-fluorophenylalanine.

7. The process according to claim 1, wherein the DNA fragment contains an anthranylic acid synthetase gene.

8. The process according to claim 7, wherein the DNA fragment can confer resistance to tryptophan analog on a microorganism.

9. The process according to claim 8, wherein the tryptophan analog is selected from 4-methyltryptophan, 5-methyltryptophan and 6-methyltryptophan.

10. The process according to claim 7, wherein the inhibition of the anthranylic acid synthetase with tryptophan or tryptophan analogs are released.

11. The process according to claim 1, wherein the DNA fragment contains a gene of 3-deoxy-2-keto-D-arabino-heptulosonate 7-phosphate synthetase, chorismate mutase, prephenate dehydrogenase or pretyrosine aminotransferase.

12. The process according to claim 11, wherein the DNA fragment confers resistance to phenylalanine, tyrosine or an analog thereof on a microorganism.

13. The process according to claim 1, wherein the DNA fragment contains a gene coding for the enzyme involved in the biosynthesis of threonine from aspartic acid to threonine.

14. The process according to claim 1, wherein the DNA fragment can confer resistance to histidine or a histidine analog on a microorganism.

15. The process according to claim 1, wherein the vector is a vector derived from a microorganism belonging to the genus Corynebacterium or Brevibacterium or a derivative thereof.

16. The process according to claim 15, wherein the vector is selected from pCG1, pCG2, pCG4, pCG11, pCE51, pCE52, pCE53, pCE54 and pCB101.

17. The process according to claim 1, wherein the microorganism belongs to the genus Corynebacterium or Brevibacterium.

18. The process according to claim 17, wherein the microorganism has an amino acid-productivity.

19. The process according to claim 17, wherein the microorganism is selected from the species Corynebacterium glutamicum, Corynebacterium herculis, Brevibacterium flavum and Brevibacterium lactofermentum.

20. A microorganism belonging to the genus Corynebacterium or Brevibacterium and containing a recombinant DNA of a DNA fragment containing a gene involved in the biosynthesis of an amino acid derived from a microorganism belonging to the genus Escherichia, Corynebacterium or Brevibacterium and a vector DNA.

21. The microorganism according to claim 20, wherein the amino acid is histidine.

22. The microorganism according to claim 21, which is Corynebacterium glutamicum K32, ATCC 39281, Corynebacterium glutamicum K49, FERM BP-464, Corynebacterium herculis K33, ATCC 39282, Brevibacterium flavum K34, ATCC 39283 or Brevibacterium lactofermentum K35, ACTT 39284.

23. The microorganism according to claim 20, wherein the amino acid is tryptophan.

24. The microorganism according to claim 23, which is Corynebacterium glutamicum K20, ATCC 39035, Corynebacterium glutamicum K31, ATCC 39280 or Corynebacterium glutamicum K37, ATCC 39285.

25. The microorganism according to claim 20, wherein the amino acid is phenylalanine.

26. The microorganism according to claim 25, which is Corynebacterium glutamicum K39, FERM P-7088.

27. The microorganism according to claim 20, wherein the amino acid is isoleucine.

28. The microorganism according to claim 27, which is Corynebacterium glutamicum K41, FERM P-7161 or Brevibacterium flavum K42, FERM BP-355.

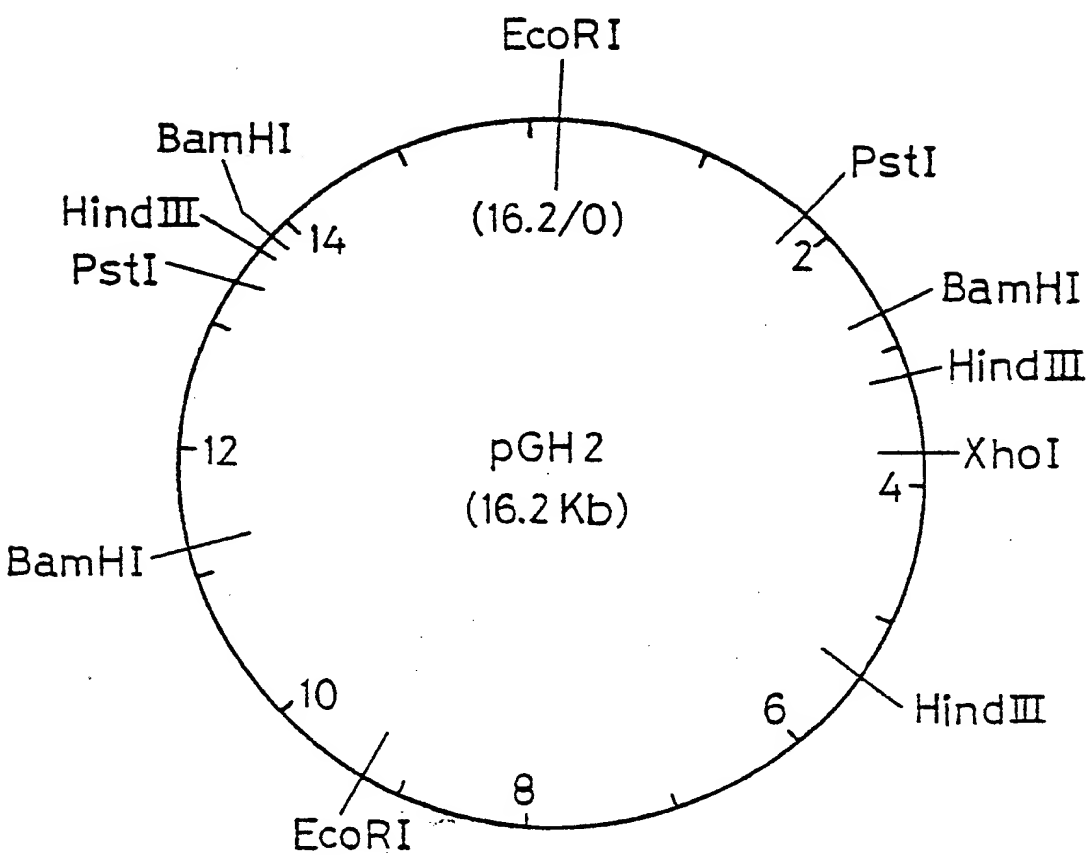
29. The microorganism according to claim 20, wherein the amino acid is tyrosine.

30. The microorganism according to claim 29, which is Corynebacterium glutamicum K44, FERM P-7163 or Corynebacterium glutamicum K45, FERM P-7164.

31. The microorganism according to claim 20, wherein the amino acid is arginine.

32. The microorganism according to claim 31, which is Corynebacterium glutamicum K46, FERM BP-356 or Brevibacterium flavum K48, FERM BP-357.

Fig. 1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PstI
2
BamHI
HindIII
XhoI
4
HindIII
6
8
EcoRI
10
BamHI
12
PstI
HindIII
BamHI
14
pGH 2
(16.2 Kb)

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2

Fig. 2

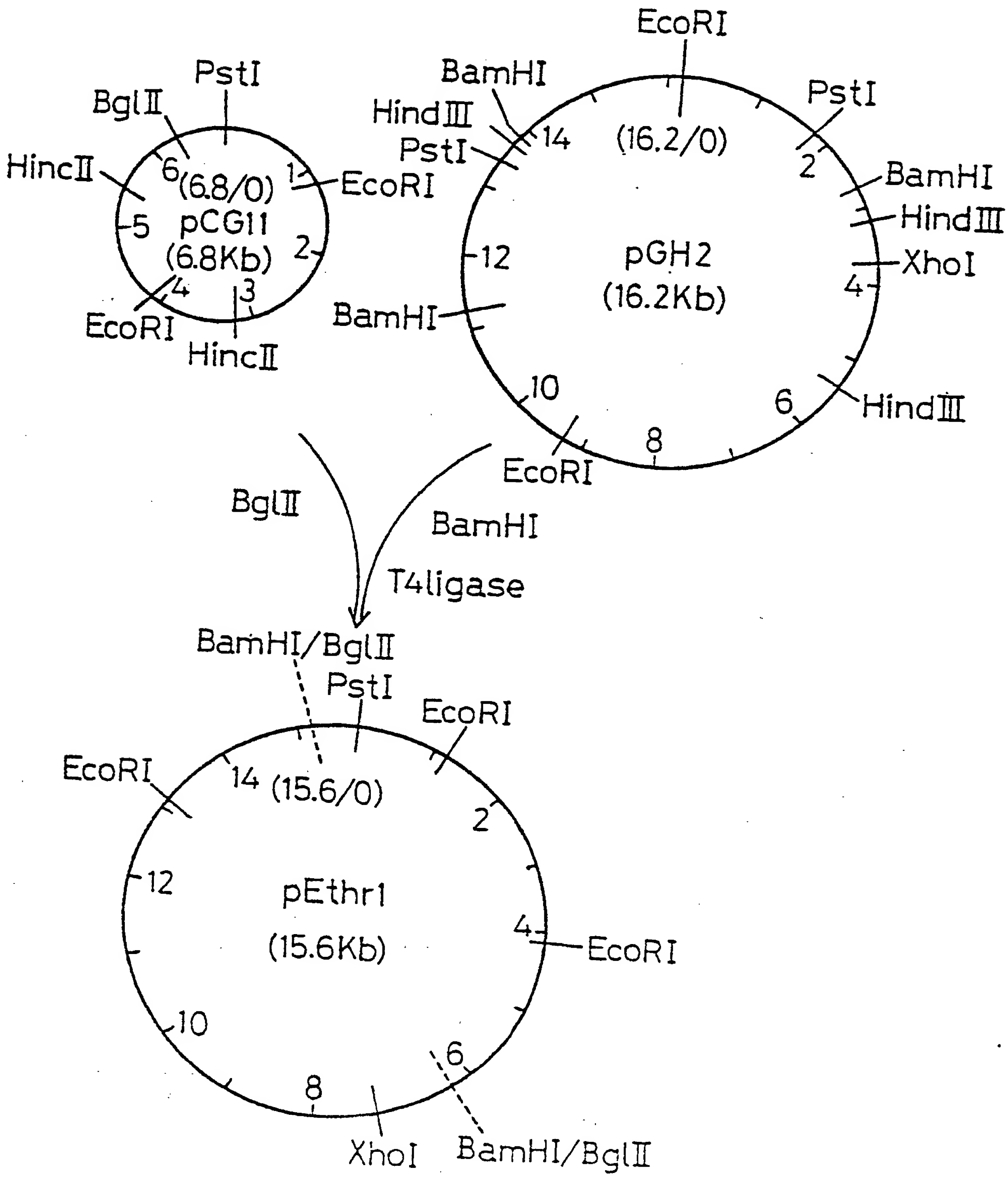
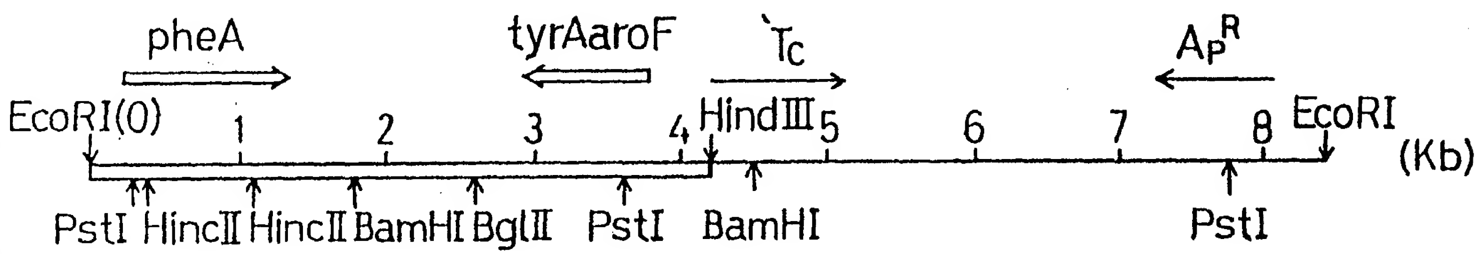


Fig. 3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PstI
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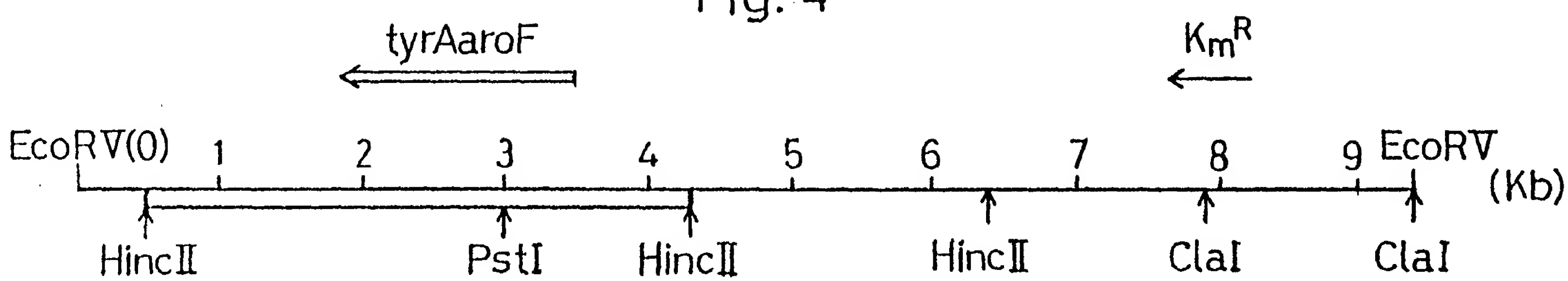
PstI

Fig. 4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K_m^R
EcoRV(0)
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2
3
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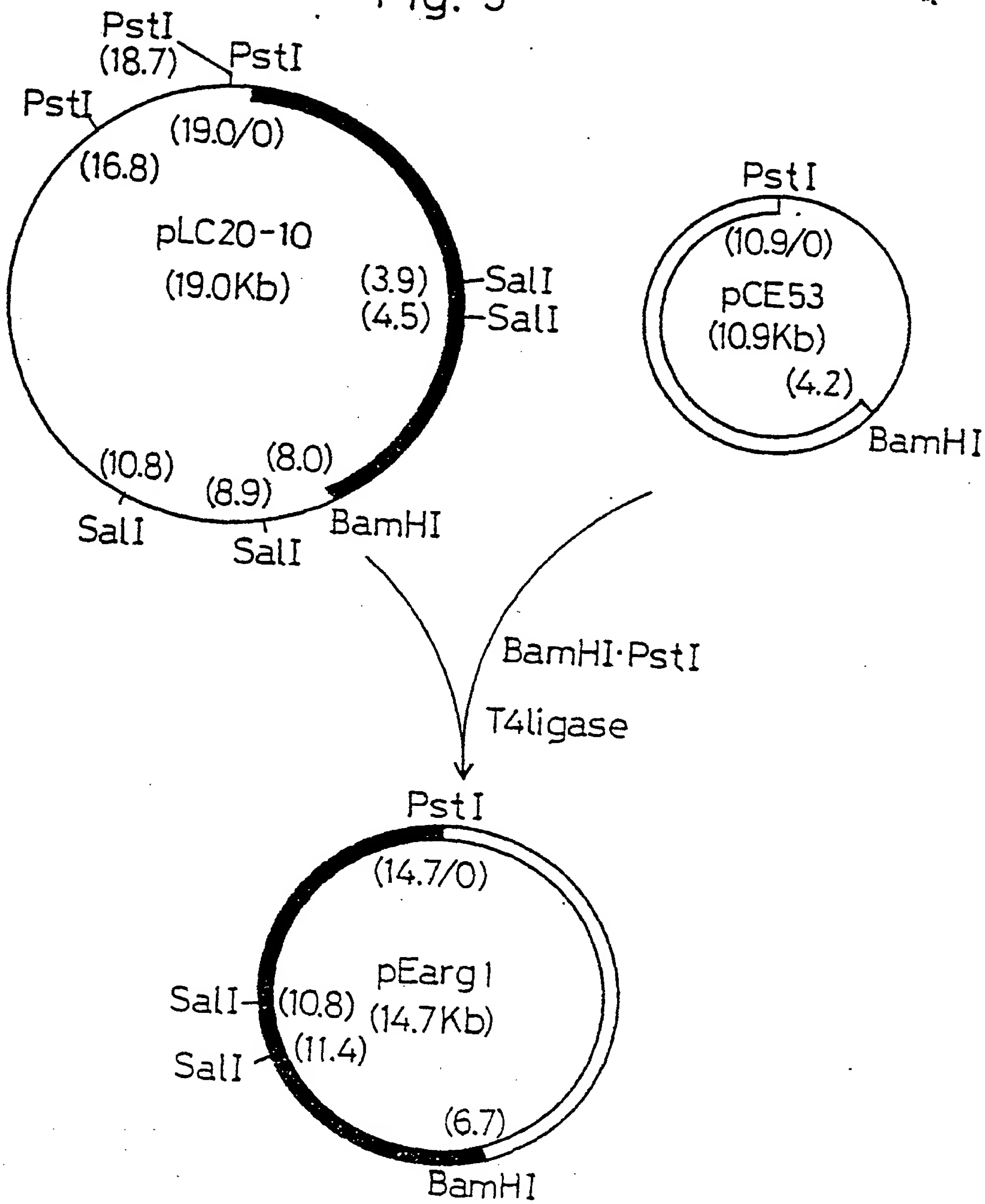
PstI
HincII
HincII
ClaI
ClaI

Fig. 5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(4.5)
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SalI
(10.8)
(8.0)
(8.9)
BamHI
SalI
SalI
PstI
(10.9/0)
pCE53
(10.9Kb)
(4.2)
BamHI
BamHI·PstI
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PstI
(14.7/0)
pEarg1
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(11.4)
SalI
(6.7)
BamHI

INTERNATIONAL SEARCH REPORT

0136359

International Application No. PCT/JP84/00047

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl^3 C12P 13/04, C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	C12N 15/00, C12P 13/04, C12P 13/06, C12P 13/10, C12P 13/22, C12P 13/24

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	Patent Laid-Open No. 148296/1981	1
X	Patent Laid-Open No. 148295/1981	1
A	Patent Laid-Open No. 5693/1982	1 - 32
A	Patent Laid-Open No. 186496/1982	1 - 32
A	Patent Laid-Open No. 186492/1982	1 - 32

* Special categories of cited documents: [15]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
May 7, 1984 (07. 05. 84)	May 14, 1984 (14. 05. 84)
International Searching Authority [1]	**Signature of Authorized Officer [20]**
Japanese Patent Office	